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A Case Study of Selected Volatile Phenols from *Brettanomyces* and Micronutrients Mn, Fe, Cu, Zn in Chianti Red Wines

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Abstract

Volatile phenols 4-Ethylphenol (EtP) and 4-Ethylguaiacol (EtG), and selected metals Mn, Fe, Cu, and Zn in Chianti red wines were determined via Head-Space Solid Phase Micro-Extraction pretreatment and Gas Chromatography-Mass Spectrometry and through Atomic Absorption Spectrophotometry. The wine samples were Chianti Classico DOCG (CdB) and Toscana IGT (CF) from seven vintages 2008-2003, 2001. The concentration for EtP was in the range 283-862 (CdB) and 155-643 μg L^{-1} (CF), whereas the content of EtG ranged 40-116 (CdB) and 11-104 μg L^{-1} (CF). The content for metals ranged 1.42-1.82, 0.93-4.68, <0.10-0.20, and 0.53-1.10 mg L^{-1} for Mn, Fe, Cu, and Zn, respectively. The concentrations for EtP and EtG have high relative values for wines from 2004 and 2007 harvests. Interestingly, Manganese concentration follows a similar trend. Data about the metals and phenols are in agreement with much care at vine/grape and fermentation-aging-refinement procedures.

Keywords: volatile phenol, brettanomyces yeast, metal, red wine analysis, AAS, GC-MS

1. Introduction

Volatile phenols like 4-ethylphenol (EtP) and 4-ethylguaicol (EtG) (Scheme 1) are among the aroma compounds that might damage the quality of wines once their contents are higher than certain threshold values. A report relevant to red Bordeaux wines and published some two decades ago claimed that the aggregate detection threshold of ethylphenols or perception limit (PL) was 426 μg L^{-1} (Chatonnet, 1992) by employing a panel of twenty tasters that followed a procedure previously constructed by the same laboratory (Boidron, 1988). The two ethylphenols are mostly produced by the yeast *Brettanomyces/Dekkera bruxellensis* (Dias, 2003). The precursors are p-coumaric acid and ferulic acid for EtP and EtG, respectively (Pollnitz, 2000). The unpleasant aromas in wines by EtP were described by a series of adjectives like: horsy, leather, smoky, medicinal, animal, etc. Similar terms were used for describing undesirable smells from EtG and a large number of publications deals with the influence by the two volatile phenols on wines. See for example Refs by Clarke (2004), Vine (2002), Goode (2005), Jacobson (2006), Licker (1999) and Zoecklein (1999). The works devoted to studying EtP and EtG in wines are many up to date. The number of hints from the search based on the key words (in the title) "volatile AND phenol AND wine" through the journals/books published by Elsevier, American Chemical Society, Wiley online library, and the American Journal of Enology and Viticulture is thirteen, one, four, and two, respectively, in the time period 2000-2012. Most of the works are relevant to microbiology issues instead of chemical ones (analytical, correlation, etc.). The content of most of the paper/book chapters shows that the interest in the field is still vivid. Nevertheless, much more efforts have to be paid especially on the determination of the phenols contents, as well as of other analytes. It has to be recalled that among the previously published papers on ethylphenols a recent article (Romano, 2009) reported that the PL by Chatonnet et al. (Chatonnet, 1992) in red Bordeaux wines was not confirmed in subsequent works (see for example Ref by Goode, 2005). In fact, wine complexity can easily influence the taster detection threshold.

We wish to report here on the selected results from the determination of EtP and EtG, and the metals Mn, Fe, Cu, and Zn in two red wines from one of the well known winemakers from the Chianti area in the Comune di Gaiole

in Chianti, Siena, Italy.

2. Method

2.1 Reagents and Materials

Ultrapure 4-ethylphenol (EtP), 4-ethylguaiacol (EtG), 3,4-dimethylphenol (Me_2P) (Figure 1) and NaCl were purchased from Sigma-Aldrich Chemical Co. (Milan, Italy). The ethanol (EtOH) used for standard solution preparations was HPLC grade (Sigma-Aldrich Chemical Co.; Milan, Italy).

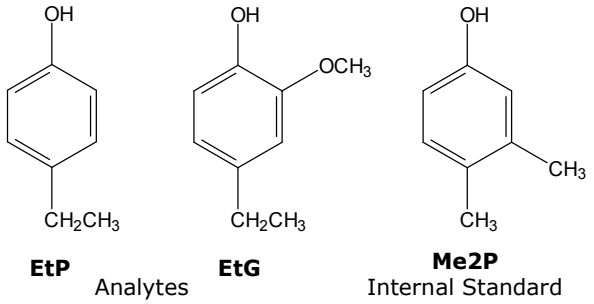

Figure 1. Structural formula for the two volatile phenols (analytes) determined in this work (4-ethylphenol, EtP and 4-ethylguaiacol, EtG) and for the volatile phenol used as internal standard in the analytical procedure (3,4-dimethylphenol, Me_2P; see text for further details)

Suprapur® mother standard solutions contained 1.000 g L^{-1} of the elements Mn and Zn and were purchased from Merck (Darmstadt, Germany). The mother standard solution of Fe and Cu were previously prepared by dissolving 0.1000 g of ultrapure metal wires (Merck) in ultrapure nitric acid (65%, Merck) and then diluted to 100 mL. Suprapur® H_2O_2 (Perhydrol® 30%) and $HClO_4$ (70 %) were also purchased from Merck.

Water for hydro-alcoholic solutions and for dilutions was of Milli-Q ultrapure type water (mqw), obtained from a Milli-W Advantage A10 system (Millipore, Milan, Italy). The ultrapure helium and acetylene gasses were from SolGroup (Milan, Italy).

Micropipettes Nichipet EX (Nichiryo, Flanders, USA), 10-100 µL and 100-1000 µL, and polyethylene Diamond D200 (200 µL) e D1000 (1000 µL) (Gilson, Middleton, USA) tips were used throughout the work.

2.2 Samples

The samples included two wine types (bottled): namely, CdB and CF. Three different bottles (same production lot) were taken from seven vintages (2008-2003, 2001). The analyses were performed in the periods July-November 2008 (2006-2003, 2001) and March-September 2011 (2007-2008).

2.3 EtP and EtG Analysis

2.3.1 Standard Solution

The mother standard solutions of volatile phenols were prepared in EtOH as 10 mg mL^{-1} and 100 µL mL^{-1} for EtP and EtG, respectively, and 10 mg mL^{-1} for Me_2P that was used as internal standard. Low content standards were then obtained by diluting the mothers with mqw (so that H_2O/EtOH = 87:13 v/v): Me_2P, 486.1 or 618.8 µg L^{-1}; EtP, 487.4 µg L^{-1} (standard additions were performed in a way to produce concentrations of the added species equal to 121.75 and 243.5 µg L^{-1}); EtG, 241.3 µg L^{-1} (standard additions, 60.25 and 120.5 µg L^{-1}).

2.3.2 Pre-Treatment and Pre-Concentration Technique

The red wine samples were pre-treated through the head-space solid phase micro extraction (HS-SPME) technique before the gas chromatography-mass spectrometry (GC-MS) quantitative analysis, in order to allow matrix simplification and analyte pre-concentration. The procedure was optimized via trial and error method on standard solutions, by starting from previous reports (Monje, 2002; Pizarro, 2007) and the guide lines from the producer (Sigma-Aldrich Co. Supelco, 2010) according to the available instruments (see just below) and to results from external private laboratory. The fiber for SPME was a polyacrylate one, 85 µm (Supelco, Milan, Italy). Vials of 2.0 cm (diameter) 4.7 cm (height) (Varian, Turin, Italy) were used. A 3 mL sample (wine) or standard hydro-alcoholic solution, was added by 1.0 g NaCl, and Me_2P (see above for concentration on the total

final volume). The vial was then hermetically sealed trough an aluminum cap equipped with a Teflon septum. The needle was inserted and the fiber exposed to the head space for 40 min (55±1°C). Stirring was applied via a 5 mm magnet at 500 rpm. It is important to note that the geometry of the system was very important as regards repeatability of the analytical determination. It was maintained fixed for all the measurements.

2.3.3 GC-MS

The instrument was a gas chromatograph GC Varian 3800 coupled with a mass spectrometer MS Saturn 2000 (Varian, Turin, Italy) available at the CIADS (Center for Analysis and Structural Determinations, University of Siena). The machine was managed and controlled via the Saturn Workstation software (Version 5.3) implemented on a Pentium IV personal computer.

The capillary column was a fused silica 30 m x 0.25 mm (Varian, Turin, Italy) with a 0.25 μm coating thickness DB5 (5% phenyl/methylpolysiloxane). The carrier gas was ultrapure He (flow, 1 mL min^{-1}). After the absorption (in the vial head space) the fiber desorbed the analytes in the GC injector for 5 min at 250°C (open split, 2 min; ratio, 25). The injector was equipped by a specific SPME inlet (internal diameter, 0.75 mm). The chromatographic oven was initially 40°C for 1 min, then was raised to 180°C at 5°C min^{-1} (hold 0.50 min) and finally was raised to 230°C at 30°C min^{-1} (hold 2 min): the total chromatographic run being 33.17 min. Before each cycle of analysis (sample "as it is" and at least two additions) the fiber was cleaned by performing at least a complete chromatographic cycle.

2.3.4 Calibration and Analysis

In order to better control the matrix effect, the standard addition method (SAM) was used for the quantitative analysis of EtP and EtG. Therefore, each quantitative determination was obtained from the analysis of the wine sample "as it is" and two more samples added by growing amount of analytes: EtP, 121.75 and 243.5 μg L^{-1}; EtG, 60.25 and 120.5 μg L^{-1}. An internal standard was also added to all the samples at a constant concentration of Me_2P (618.8 μg L^{-1}).

The chromatograms showed three peaks at 14.12 and 17.15 min for EtP e EtG, respectively, and at 14.91 min for Me_2P, retention times. The two analytes had well isolate peaks, while the Me_2P peak showed a partial overlap with an other peak that was not of interest for the present study. However, the use of an MS as detector allowed a good quantification of the internal standard. The integration was carried out on the basis of the ions 107, 107 and 152 m/z respectively for EtP, Me_2P and EtG.

2.4 Analysis of Metals

2.4.1 Standard Solutions

The standard solutions for calibration purposes and for standard additions were obtained by diluting the mother solutions as purchased, with mqw added by ultrapure nitric acid (0.2% HNO_3).

2.4.2 AAS

The analyses were carried out with a flame atomic absorption spectrophotometer Perkin-Elmer 5000 (Perkin-Elmer, Monza, Italy) for Mn, Fe, Cu, and Zn. The flame was fueled with an acetylene-air mixture. The lamp used for the analyses was a multi-element hollow cathode lamp (combinations: Cr, Mn, Fe, Cu, Ni and Zn, Perkin-Elmer, Monza, Italy). The absorbance recorded for each sample was an average of ten readings. The wavelength lines used in the analyses are 279.5, 248.3, 324.7, and 213.9 nm for Mn, Fe, Cu, and Zn, respectively.

2.4.3 *Pre-Treatment Technique, Calibration and Analysis*

In order to determine the most efficient method for analysis of the metals, four different treatments were explored for the samples: (i) sample analyzed "as it is" (without any treatment); (ii) mineralization procedure by treating 10 mL of wine with 0.5 mL each of HNO_3 (65%) and H_2O_2 (30%); (iii) mineralization procedure treating 10 mL of wine with 0.5 mL each HNO_3 (65%) and $HClO_4$; (iv) standard addition method (SAM). All four procedures were used for the analysis of Mn, Fe, and Cu, via flame-AAS in a selection of samples. For the analyses of samples "as it is" of Mn and Fe, no addition of standard solution to the wine was necessary. Some addition (Cu, 0.2 mg L^{-1}) of standard was necessary for analyses of copper (for (i), (ii) and (iii) procedures), because the absorption for the untreated wine resulted close to the limit of detection (LOD_{Cu}, 0.10 mg L^{-1}). In the case of procedures (ii) and (iii) the analyses for each metal were carried out twice: the first determination was carried out after a hour of digestion and the second one, after twenty-four hours digestion. When procedure (iv) was carried out, the standard additions for the analysis of Mn were by 0.50, 0.75, and 1.00 mg L^{-1} to each of the selected samples. Table 1 reports comparison of data for Mn for selected samples. Based on this comparison as

well as on those for Fe and Cu, it was concluded that analysis of sample "as it is" (i) and SAM (iv) provide the most repeatable and time efficient methods. The additions for analysis of Fe were 1.00 and 1.50 mg L^{-1}, whereas those for Cu were 0.20, 0.30, and 0.40 mg L^{-1}.

The concentration of Zn was also analyzed in all the samples. For the analysis of samples of wine "as it is" some 1:5 dilution of the samples was necessary. The same dilution factor was used in the standard addition procedure, along with additions of 0.10, 0.20, and 0.40 mg L^{-1}.

Table 1. Comparison of analytical methods used in this work for the quantitative determination of Mn in selected red wine samples. The values are in mg L^{-1}

Sample	Mn (mg/L)					
Method	i	ii (1h)	ii (24h)	iii (1h)	iii (24h)	iv
CdB01-1	1.71(1)	1.74(2)	1.62(1)	1.75(1)	1.59(1)	1.45(1)
CdB01-2	1.66(1)	1.74(1)	1.64(1)	1.79(1)	1.61(1)	1.39(1)
CdB06-1	1.72(1)	1.81(1)	1.68(1)	1.87(1)	1.64(1)	1.49(1)
CdB06-2	1.72(1)	1.81(1)	1.70(1)	1.85(1)	1.64(1)	1.51(1)
CdB06-3	1.74(1)	1.81(1)	1.68(1)	1.81(1)	1.56(1)	1.46(1)
CF01-1	1.77(1)	1.85(1)	1.68(1)	1.89(2)	1.67(1)	1.49(1)
CF01-2	1.78(1)	1.81(1)	1.65(2)	1.85(1)	1.67(1)	1.48(1)
CF01-3	1.75(1)	1.85(1)	1.68(1)	1.89(1)	1.70(1)	1.54(1)
CF03-1	1.72(1)	1.81(1)	1.68(1)	1.87(1)	1.67(1)	1.48(1)
CF03-2	1.74(1)	1.85(1)	1.70(1)	1.89(1)	1.65(1)	1.50(1)
CF03-3	1.75(1)	1.87(1)	1.73(1)	1.93(1)	1.68(1)	1.52(1)

2.5 Brettanomyces Yeasts Analysis

The analyses of *Brettanomyces* yeasts were done by following procedures previously reported in literature (Minacci, 2005; Cavazza, 1992; Rossini, 2003). The analyses were carried out on agar-culture medium of the type WL (Wallerstein Laboratory; Green, 1950) differential Agar+Caf (cloramfenicolo) containing hydrolyzed enzymatic casein (5 g L^{-1}), yeast extract (4 g L^{-1}), magnesium sulfate (0.25 g L^{-1}), dextrose (50 g L^{-1}), potassium phosphate (0.55 g L^{-1}), potassium chloride (0.425 g L^{-1}), calcium chloride (0.125 g L^{-1}), iron chloride (0.0025 g L^{-1}), manganese sulfate (0.0025 g L^{-1}), green cresol bromide (0.022 g L^{-1}), actidione (0.004 g L^{-1}), chloramphenicol (0.05 g L^{-1}) and agar (20 g L^{-1}). The liquid culture medium was the specific SNIFF BRETT® (patented by IntelliOeno, www.intellioeno.com, containing ethyl-phenols precursors).

For each sample six WL differential agar plates were used by using two different techniques: (i) seeding on plate (three plates, 1 mL of wine each plate); (ii), filtering membranes, FM (three plates, 100 mL of wine each plate). Meanwhile, the analyses on liquid culture medium were carried out on three media for each sample, by inoculating 20 mL of wine. All tests were performed in triplicate.

3. Results

The concentrations of the volatile phenols EtP and EtG in the red Chianti wines are reported in Table 2, whereas the concentrations of selected metals in the same wines are listed in Table 3. The procedures used in this work are reliable as shown from the closeness of data obtained from this laboratory and from an independent private laboratory specialized on analysis of beverages and food.

3.1 CdB Wines

3.1.1 Phenols

The CdB wines from vintages 2008-2003, 2001 have weighted mean contents of EtP (CEtP) ranging 283-862 µg L^{-1}. The analysis of data reveals that the content for the wines from 2006 vintage (283 µg L^{-1}) when analyzed the year 2008 is small and below the perception limit (PL) 426 µg L^{-1} claimed previously for red Bordeaux wine (Chatonnet, 1992). Subsequent works by others revealed that PL is much influenced by the presence of other components (Romano, 2009). It has to be noted that the relative weighted standard deviations from the present

work are below 10% (mean value) and usually better than those from corresponding determination performed by using extraction methods followed by GC-MS techniques (Pollnitz, 2000).

Table 2. Concentrations for the volatile phenols 4-ethylphenol (EtP) and 4-ethylguaiacol (EtG) (μg L^{-1}) as determined via HS-SMPE/GC-MS (see text for details). The concentrations are given as the weighted means[a] on usually three independent determinations on bottles from the same lot. The values in parenthesis ( ) are the weighted estimated standard deviations[a]. LOD (limit of detection) values are: 50 and 5 μg L^{-1}, for EtP and EtG, respectively

Sample	EtP	EtG	Sample	EtP	EtG
CdB01	454(9)	69(1)	CF01	586(14)	71(1)
CdB03	718(17)	116(2)	CF03	580(13)	68(1)
CdB04	862(20)	80(1)	CF04	625(15)	78(1)
CdB05	771(18)	110(2)	CF05	643(15)	104(2)
CdB06	283(7)	40(1)	CF06	252(6)	55(1)
CdB07	668(16)	106(2)	CF07	202(5)	12(1)
CdB08	371(9)	41(1)	CF08	155(4)	11(1)

[a] Weighted mean and weighted estimated standard deviations are computed as follows: $\bar{x}_w = \sum_{i=1}^{N} w_i x_i / \sum_{i=1}^{N} w_i$, where x_i = i-th observation, $w_i = ABS(1/((x_i - \bar{x})/\bar{x})$, $\bar{x} = \sum_{i=1}^{N} x_i / N$ and $esd_w = SQRT\{[\sum_{i=1}^{N} w_i (x_i - \bar{x}_w)^2] / D\}$, where: $D = SQRT\{(N'-1)/N' \sum_{i=1}^{N} w_i\}$, is the number of observations, N' is the number of non-zero weights.

Starting from the small concentration for year 2006 samples, CEtP increases significantly going backwards to the years 2005 and 2004 (771 and 862 μg L^{-1}, respectively), and it decreases again for the years 2003 and 2001 (718 and 454 μg L^{-1}). It has to be noted that the year 2007, CEtP increases up to 668 μg L^{-1} and decreases again to 371 μg L^{-1} for the vintage 2008.

The concentrations of EtG (CEtG) for the years 2006-2003, 2001 are 40, 110, 80, 116, 69 μg L^{-1}. The CEtG for wines from 2004 vintage is significantly below the values for the vintages of the years 2005 and 2003. Anyway, the gross trend is similar to that for CEtP, even if the year 2007 and 2008 (106 and 41 μg L^{-1}, respectively) are considered.

It has to be noted that the RP (CEtP:CEtG) factor is 9.0, 6.3, 7.1, 7.0, 10.8, 6.2, 6.6 (RP_{mean}, 7.5) for the CdB wines from years 2008-2003, 2001 respectively. Noticeably, the RP values for all the vintages examined but 2004, are smaller than 10, this latter being considered as a regular average for Bordeaux red wines (Chatonnet, 1992). The RP_{mean} is 6.6 when just the CdB wines from 2007, 2006, 2005, 2003, 2001 are taken into account and RP_{mean} is 7.0 when also the 2008 vintage is considered. Other RP values previously reported in literature for other wines are: Shiraz, RP_{mean} 13.4 (495:37), Cabernet-Sauvignos, RP_{mean} 10.1 (771:76), $Nebbiolo_{mean}$ RP 7.5 (368:49), Pinot-Noir 2.4_{mean} (120:50) as found by analyzing 30, 13, 16 different wines (Goode, 2005); Shiraz, RP_{mean} 9.2 (605:66); Cabernet-Sauvignos, RP_{mean} 10.1 (1250:124), Pinot-Noir, RP_{mean} 3.5 (338:97) as found by analyzing 21, 18, 13 different Australian wines (Pollnitz, 2000). Noteworthy, this latter article reports RP values in the ranges (for the same wines): 3.6-22.9 (186:51-709:31), 3.8-17.4 (1130:295-2450:141), 1.4-6.0 (32:23-169:28), respectively. The RP_{mean} value for all varieties is 8.0.

Table 3. The content of selected metals Cu, Mn, Fe, Zn (mg L^{-1}) as determined via FAAS (see text for details). The concentrations are given as the weighted means on usually three independent determinations on bottles from the same lot. The values in parenthesis ( ) are the weighted estimated standard deviations. LOD (limit of detection) values are: 0.3, 1.0, 0.10, and 0.1 mg L^{-1} for Mn, Fe, Cu, and Zn, respectively

Sample	Mn	Fe	Cu	Zn
CdB01	1.42(1)	4.68(5)	0.16(1)	0.93(2)
CdB03	1.67(1)	2.41(4)	0.20(2)	0.71(4)
CdB04	1.71(1)	1.65(1)	<LOD	0.64(4)
CdB05	1.58(1)	1.80(1)	0.10(1)	0.77(4)
CdB06	1.49(2)	1.61(1)	0.14(1)	0.77(3)
CdB07	1.82(4)	2.8(1)	<LOD	1.10(4)
CdB08	1.52(3)	1.37(5)	0.15(1)	0.89(2)
CF01	1.50(2)	3.3(2)	0.12(1)	0.85(6)
CF03	1.50(1)	3.6(1)	0.14(1)	0.77(3)
CF04	2.03(1)	1.57(2)	0.12(1)	0.66(3)
CF05	1.48(1)	1.89(1)	0.17(2)	0.68(2)
CF06	1.57(1)	0.93(1)	0.15(1)	0.53(3)
CF07	1.52(1)	1.81(5)	0.15(2)	0.80(3)
CF08	1.26(1)	1.03(1)	0.14(2)	0.63(3)

These data show a large variability within the same variety and on passing from a variety to another for both EtP and EtG. Although some claim about a tendency towards rising of *Brettanomyce*s in wines with time have been reported (Goode, 2005) this is but just a conjecture in our opinion. As it is commented above, the data from the present work do not confirm those claims, in fact, while an increase in the concentrations of EtP and EtG was generally detected from 2001 up to 2004 vintages, then a decrease was found for 2005 and more significantly for 2006 vintages. On examining the extensive batches of data from Pollnitz et al. (Pollnitz, 2000) no any regular trend towards significant increase could be found for the vintages from the years 1986-1997 (Cabernet-Sauvignon, Merlot, Pinot-Noir and Shiraz in Australia). We believe that several factors contribute in defining the total and relative content and EtP and EtG in wines: variety of vines, area and *terroir*, vintage (climate conditions), wine making conditions (treatment with SO_2 at grape crushing, hygiene conditions at cellar/wood), the content of precursor compounds, conditions that favor specific enzymes that act in the chains of EtP/EtG production. In case of wines that undergo micro-filtration after refinement in wood and before bottling (so to remove *Brettanomyces* colonies) the content of volatile phenols presumably decreases with times owing to their (photo) chemical instability and owing to escaping from the cork's porous. The photochemical instability of phenols could be a not negligible factor. In fact, a recently published paper (Clark, 2011) reports that tartrate undergoes more significant photo-degradation when linked to Fe^{III}, than when free. Thus, on the basis of the well established affinity of Fe^{III} towards phenols one can argue that even photo-degradation of phenols is probably more pronounced when they are in the coordination sphere of iron cations. Other redox active cations (like Mn^{III}/Mn^{II}, and Cu^{II}/Cu^{I}) could be also responsible for activation of photo-chemical reactions on phenols in wines at some extent.

3.1.2 Metals

The content of Mn (CMn) for CdB wines for the vintages 2008-2003, 2001 ranges 1.42-1.82 mg L^{-1}, the maximum value being the concentration for the year 2007 (Table 3) (CMn_{mean}, 1.60 mg L^{-1}). Therefore the gross trend is similar to that found for the content of phenols. The CMn:CEtP ratio is 4.1, 2.7, 5.3, 2.1, 2.0, 2.3, 3.1 for CdB wines from vintages 2008-2003, 2001, respectively, showing that small increases of Mn cause larger increase for EtP. Further comments on Mn, as related to phenols are reported below.

The content of Fe (CFe) for the vintages 2008-2003, 2001 is 1.37, 2.8, 1.61, 1.80, 1.65, 2.41, 4.68 mg L^{-1}. It has to be noted that CFe for wines from vintages 2006, 2005, 2004 is low and average 1.69(10) mg L^{-1}, whereas CFe

for the years 2003 and 2001 is much higher, and trend for wines from the years 2001, 2003 and 2004 is linear (R^2 = 0.992, P = 0.05). On excluding the year 2007, the data are in agreement with decreased up-take of iron from the vines and with reduced treatments with iron-containing additives to wine at fining/aging stages. During the last half a dozen years most of cellar tools and equipments that had iron, bronze or brass components were replaced by stainless still or polymeric material in order to avoid the transfer of heavy metal ions to must and wine. For instance, was proved that the old metal pumps for usage in the cellars caused significant leaching especially from welding (Crowe, 2011).

The content of Cu (CCu) for the vintages 2008-2003, 2001 ranges < LOD (0.10 mg L^{-1})-0.20 mg L^{-1}, and averages 0.14 mg L^{-1}. It has to be remarked that for all the wines the content of the metal is very low when compared to other red wines from the same area (Chianti) or from other areas in Italy or from other countries (Tamasi, 2010). The maximum allowable concentration for Cu in wines under the Italian and EC regulation is 1 mg L^{-1} (Commission Regulation European Community, 2000). No any fitting could be easily envisaged for the data; notwithstanding, it has to be noted that for the vintages 2007, 2005 and 2004 CCu was very low, as opposite to CEtP and CEtG.

As regards Zn the content for the vintages 2008-2003, 2001 is 0.89, 1.10, 0.77, 0.77, 0.64, 0.71, 0.93 mg L^{-1} (CZn_{mean}, 0.83 mg L^{-1}). It has to be noted that CZn decreases from 0.93 mg L^{-1} the year 2001 to 0.64 for the year 2004 increasing then again up to 1.10 mg L^{-1} in the year 2007 and decreasing for the 2008 (0.89 mg L^{-1}).

3.2 CF Wines

It is important to underline that the blending for the CF wine changed on 2007 from Sangiovese 70% and Merlot 30% blend to Merlot 100%. Not withstanding, some comments about data relevant also 2007 and 2008 vintages can be attempted.

3.2.1 Phenols

The concentration of 4-ethylphenol (CEtP) for the wines from the vintages 2008-2003, 2001 are 155, 202, 252, 643, 625, 580, 586 μg L^{-1} and shows the highest value for years 2005, but the wine from year 2004 is high, too, and within the weighted standard deviations.

A similar trend was found for the CEtG for the wines from the same vintages: 11, 12, 55, 104, 78, 68, 71 μg L^{-1}; the content for the year 2005 being the maximum, whereas that for the year 2008 the minimum. It can be noted that the last two vintages 2007 and 2008 seem to be very different from the previous ones, according to the new blending that characterizes this wine.

The RPs (CEtP:CEtG ratio) are 14.1, 16.8, 4.6, 6.2, 8.1, 8.5, 8.3 for the seven examined vintages. The trend for the RP values for CF wines has significant differences when compared to those for CdB wines. In fact, the values for the years 2004, 2003, and 2001 average 8.3; instead the values for 2006 and 2005 are significantly smaller 4.6 and 6.2, respectively. Finally the higher values were fund for the years 2007-2008. The value of CP (CEtP+CEtG) is at a minimum for the year 2006 (lots analyzed Fall 2008) and the content of EtP (the more unpleasant species) is comparatively decreasing faster the years 2004-2006. Considering the more recent vintages (2007-2008), also, the value of CP decreases up to 166 μg L^{-1} for the year 2008 (minimum; lot analyzed Fall 2011) and the content of EtG becomes very low (11 μg L^{-1}). These findings are opposite to the claims of current increases of *Brettanomyces* (Goode, 2005), instead they are suggestive of more accurate wine making processes.

3.2.2 Metals

The CMn values from vintages 2008-2003, 2001 are 1.26, 1.52, 1.57, 1.48, 2.03, 1.50, 1.50 mg L^{-1}. The trend for CMn is similar to that found for CdB wines. While the years 2007-2005, 2003 and 2001 have almost the same content of Mn (average 1.51 mg L^{-1}), CMn for the year 2004 is much higher, whereas the value for the year 2008 is the lower.

The values for CFe in CF wines for the vintages 2008-2003, 2001 are 1.03, 1.81, 0.93, 1.89, 1.57, 3.6, 3.3 mg L^{-1}. As found for CdB blend, CFe for vintages 2008-2004 is low and in the range 0.93-1.89 mg L^{-1}, whereas the contents of iron for the years 2003 and 2001 are much higher.

The values of CCu for CF blend for the vintages 2008-2003, 2001 are 0.14, 0.15, 0.15, 0.17, 0.12, 0.14, 0.12 mg L^{-1}. Mean value for CCu (0.14 mg L^{-1}) is very low with respect to MAC_{Cu} (1 g L^{-1}) and compares well with that found for CdB samples. When compared to other red Chianti wines from the same area the CF (and CdB, too) samples have very low values for CCu (Tamasi, 2010).

The content of Zn for CF wines for the vintages 2008-2003, 2001 ranged 0.53 (year 2006)-0.85 (year 2001) mg

L^{-1}. The values suggest that the attention to safe production is constant for the tme period investigated taking low the treatments based on anti-fungus zinc products for vines.

3.3 Microbiology

All the wine samples did not reveal measurable colonies of *Brettanomyces* from agar cultures. The liquid cultures did not give positive responses, too. These facts, can be explained on the basis of micro-filtration treatments of wines after aging in oak barrels and before bottling. The absence of colonies of the yeast in the bottles guarantees against an increase of volatile phenols for wines in the market; on the contrary, the CEtP and CEtG values should decrease owing to (photo) chemical decomposition and slow release from the porous of corks.

The wines under control for *Brettanomyces* were not micro-filtered before bottling. Addition of sulfur dioxide at bottling probably caused yeast killing, but that could not be experimentally proven.

4. Discussion

This work revealed that the content of EtP and EtG for two blends of red wines produced in the Chianti area (vintages 2008-2003 and 2001) have high values for the years 2005 and 2004. The concentration for EtP was 771 and 862 mg L^{-1} (CdB blend), and 643 and 625 mg L^{-1} (CF blend), those two years. Owing to the strong bouquet commonly found for red Chianti wines from Sangiovese vine variety, the typical unpleasant smell/taste from those volatile phenols could not be revealed (by a panel of twelve tasters), even for the wines from vintages 2005 and 2004. This trend of volatile phenols as function of time is difficult to be explained because vine varieties (Sangiovese for both blends), *terroir* (constant for each blend within the time period of harvests), wine making processes from grape growth ahead (treatments of vines and grape on vines, grape crushing, fermentation, wine aging and refinement procedures) were almost the same in the time period examined. A difference in climate can tentatively be identified as a possible factor for the trend found in this work, because different weather conditions can favor differences in population of yeasts on the skin of grapes. The musts/wines probably were treated with lesser amounts of SO_2 the years 2004 and 2005 because grapes were affected by smaller amounts of molds/yeasts (better weather conditions, smaller risks of infection; SO_2 is a strong scavenger for bacteria, molds and yeasts) (Zoecklein, 1999).

It has to be noted that once analyzed the Summer-Fall 2008 the samples did not show any appreciable content of *Brettanomyces* colonies. Thus, production of volatile phenols via *Brettanomyces* must have been stopped before bottling, presumably after removing the wines from wood barrels.

Finally, it has to be recalled that the high concentrations for Mn were also detected for the CdB and CF wines from 2005 and 2004 vintages. One can claim that *Brettanomyces* yeasts responsible for the synthesis of EtP and EtG, are more and more efficient in media with larger CMn.

This would suggest that the yeast contains manganese-enzymes for the synthesis of volatile phenols. Of course, we cannot state at an acceptable significance that the presence of manganese facilitates the biosynthesis of volatile phenols. In fact, (first) this work is based on a small set of data, (second) no any report was published on the positive effect of manganese for *Brettanomyces* growth. Notwithstanding, we wish to recall two points that are in agreement with possible influence of manganese on the yeast: (i) the WL culture media must contain manganese (although at trace concentration, 0.0025 g L^{-1}) (Fugelsang, 2007), (ii) manganese is very important for growth for a fundamental yeast for wine, *Saccharomyces cerevisiae* (Cizewski Culotta, 2005), (iii) some decarboxylase derivatives (oxalate and oxaloacetate) require manganese as activator/catalyzer of reactions (Tanner, 2001; Labrou, 1999). Therefore, the following would be of interest for throwing some light on the issue: first, expand the analyses for volatile phenols and manganese in large number of red wine samples; second, investigate the influence of metals and manganese in particular, on microbiology of *Brettanomyces*.

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