Optimization of a micro-scale fermentation set-up: repeatability and comparison with larger-scale red wine fermentation

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Assessing tannins in grapes remains difficult and sometimes it is insufficient to predict what will be extracted into wine. Therefore, research in viticulture tends to perform small-scale fermentations. The aim of this study was to develop and optimize a micro-scale fermentation set-up (200 g of berries) with the potential to increase the repeatability of fermentation replicates mimicking the mechanical cap management similar to standard procedures. A vineyard was established in 2003 at Hochschule Geisenheim University (Germany) with a rootstock trial (section A) using *Vitis vinifera* L. cv. 'Pinot Noir' grafted onto 17 rootstocks and a variability trial (section B) with 'Pinot Noir' grafted on SO4. Repeatability of the micro-scale method averaged at 12.1 % for anthocyanins, improved with increasing maturity (5.1 % to 9.1 %) a range between 4.2 % to 12.7 % was achieved for tannins, thus proving that the micro-scale winemaking method was repeatable. Though tannin concentration found in micro-scale fermentations was higher by 34 %, it was linearly correlated to the amount extracted during higher-scale fermentations (50 kg, 400 kg) (R^2 = 0.86, n = 23), argumenting for the reliability of the micro-scale method used for anthocyanins (R^2 = 0.89, n = 23). Ultimately, combined with berry analysis, this system could contribute to a better understanding of the influence of vineyard management on the extraction of phenolic compounds into wines.

Keywords: micro-scale winemaking, red wine, extraction, Pinot Noir, phenolics

Optimierung eines Maischegärungsverfahrens im Mikromaßstab: Wiederholbarkeit und Vergleichbarkeit mit Standardverfahren. Tannine sind eine Gruppe von sekundären Inhaltsstoffen, die in der Beerenhaut und in Kernen der Trauben vorliegen. Die Beurteilung der Konzentration in Beeren ist teils unzureichend, um die später im Wein extrahierte Tanninmenge im Wein vorherzusagen. Deshalb müssen Versuche mit weinbaulicher Fragestellung auch einen Weinausbau mit Maischegärung der einzelnen Varianten mit einplanen. Das Ziel dieses Projekts war, die Vergleichbarkeit der Maischegärung im Kleinst-oder Mikromaßstab (200 g Beeren) mit den Standardverfahren im Großen zu prüfen und die Wiederholbarkeit von Gärverläufen zu untersuchen. Die Untersuchungen wurden in einer Rebfläche der Hochschule Geisenheim (Deutschland) an Spätburgunder-Reben, die 2003 gepflanzt wurden, durchgeführt. Einem Ansatz lag ein randomisiert angelegter Unterlagenversuch mit 17 Unterlagen zugrunde. Die Untersuchungen des zweiten Teils fanden in der gleichen Fläche bei der Rebsorte ‚Blauer Spätburgunder' auf der Unterlage SO4 statt. Es konnte gezeigt werden, dass die Wiederholbarkeit der Methode für Anthocyane im Durchschnitt bei 12,1 % lag, während sie sich mit zunehmender Reife verbesserte (5,1 % bis 9,1 %). Für Tannine wurde eine Bandbreite zwischen 4,2 % und 12,6 % erreicht, was die Wiederholbarkeit dieser Mikromaßstab-Methode beweist. Obwohl die Tanninkonzentration in Weinen aus dem Mikromaßstab um 34 % höher war als in denen des Makromaßstabs (mittlerer Maßstab: 50 kg; großer Maßstab: 400 kg), konnte eine lineare Korrelation für Tannine (R^2 = 0,86, n = 23) und Anthocyane (R^2 = 0,89, n = 23) zwischen den Vergleichen bestimmt werden. In Kombination mit der Beerenanalyse könnte dieses System letztendlich dazu beitragen, den Einfluss der Weinbergbewirtschaftung auf die Extraktion von Phenolen in Wein besser zu verstehen.

Schlagwörter: Maischegärung Mikromaßstab, Rotwein, Extraktion, Spätburgunder, Phenole

Proanthocyanidins (tannins) are a class of components whose composition in wine is difficult to predict based upon fruit measurements (Adams and Scholz, 2007). Due to the complexity and the variability of a large number of environmental, chemical, technological, and biological factors which impact on the final quality of the wine, research in viticulture tends to rely on small-scale productions, allowing well-controlled conditions (Rossouw et al., 2012). A method for small-scale fermentations (3.5 kg) with a 4 l fermenter was published (Sampaio et al., 2007), thoroughly described (Benito et al., 2011) and is widely used for viticultural and enological studies. Indeed, this method was helpful in evaluating microbiological aspects (Lopes et al., 2002), enological factors (Cerpa-Calderon and Kennedy, 2008; Geffroy, 2020) and viticultural factors (Diago et al., 2013; Gil et al., 2015). Some adapted the method to larger scales like 8 l tanks (6 kg batches; Gil et al., 2012), 25 l tanks (15 kg batches; Pascual et al., 2016) or 50 l tanks (75 kg batches; Schmidt et al., 2009) and some explored the effect of scale (Sampaio et al., 2007; Lopes et al., 2002; Schmidt et al., 2009; Rossouw et al., 2012).

Their data show that extrapolation of laboratory datasets to real commercial conditions can be justified (Rossouw et al., 2012) with a relative similar temperature distribution (Schmidt et al., 2009) and microbial flora (Lopes et al., 2002). However, there is much criticism about the practical application of such micro-scale fermentation set-ups as a useful tool for red winemaking. Our aim was to develop and optimize an even smaller micro-scale fermentation set-up (100 berries). No evidence of the actual repeatability of such small-scale fermentation methods has been found. We suggest that by implementing a mechanical cap management similar to standard procedures in the micro-scale fermentation set-up, the repeatability of the method for phenolic extraction would be suitable. Furthermore, there is still doubt about the relevance and reliability of such a micro-scale fermentation set-up. Indeed, previous work indicates that tannin extraction may be lower (by 36 %) than in commercial fermentations of the same fruit with similar skin contact times (Sampaio et al., 2007). We propose that by increasing the post-fermentation maceration period for the micro-scale fermentation method its phenolic composition would be comparable to larger-scale fermentation.

Material and Methods

Micro-scale fermenter design

An effort was made to keep the fermenter design as simple as possible. Fermenters were created from jam jars with lids implemented with air-locks and a stainless steel screen to plunge down the pomace (Fig. 1). The micro-scale fermenter is a glass jar (250 ml) with a twist-off cover top for food purpose perforated in its center, where the air-lock is inserted. An air-lock (Duplex 1, Schliessmann Kellerei-Chemie GmbH, Schwäbisch Hall, Germany) filled up with water was plugged on a rubber bung and used to seal off the whole system. A wine cap punch down tool was created out of a perforated stainless-steel plate for the bottom disc of 10 cm in diameter with 25 small holes and a handle which was embedded in the air-lock system. The punch down tool would remain in each fermentation vessel during the fermentation process to keep the cap immersed. To avoid light exposure the jars were covered with aluminium foil. However, an investigation showed no significant differences whether the surrounding was covered or without the aluminium foil (data not shown).

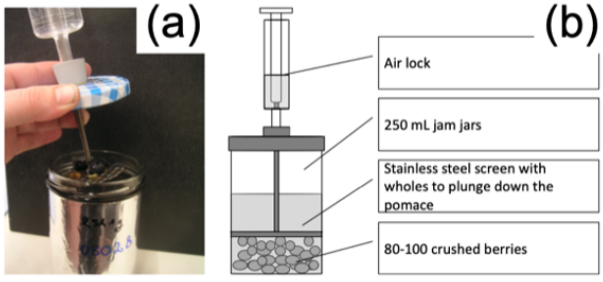

Fig. 1: Micro-scale fermenter as photograph (a) and scheme (b)

Micro-scale winemaking

Around 200 g of berry material (corresponding to 100 berries) was destemmed, crushed lightly by hand and transferred to the micro-scale fermenter. Potassium metabisulfite solution (SO_2) mixed with water was prepared to have a 5 % solution and 50 mg/kg of SO_2 was added to the mash. A subsample of 400 µl of juice was taken to evaluate primary compounds with Fourier-transform infrared spectroscopy (FTIR; OenoFoss™, FOSS, Hillerød, Denmark) providing results for organic acids, total acidity, pH and total soluble solids. The weight of each part of the micro-scale fermenter was recorded in order to evaluate the weight of the mash.

After calculating the weight of all fermentation units, a solution of inactive dry yeast Oenoferm® Klosterneuburg (*Saccharomyces cerevisiae*; Erbslöh AG, Geisenheim, Germany) was prepared and rehydration took place in around 4 ml water in a 15 ml falcon tube at 20 °C, during 20 min, mixing every 5 min. The must was inoculated with 250 mg/kg with an Eppendorf pipette and final weight was recorded. Fermentation took place in a temperature-controlled room for six days on skins and the cap was manually punched down three times daily at approximately six-hour intervals. Fermentation temperature was maintained between 20 and 25 °C by adjusting the room temperature. Our investigations showed that tannin extraction was limited when punching the pomace only once (188.3 ± 25.9 mg/l) compared to twice (286.4 ± 47.7 mg/l) or three times a day (256.9 ± 17.1 mg/l). Weight and temperature were recorded at least daily. Fermentation was considered completed when weight was stable (differences less than 0.5 %) and lasted around one week.

At that point the stainless-steel screens were removed together with the air-lock system and the fermenters were sealed with a rubber bung. A two-week post-fermentation maceration period followed and the weight of the fermenters was then again recorded. Our investigations showed that tannin extraction was not completed after four days post-maceration (201.93 ± 30.25 mg/l) compared to an extended maceration of ten (241.07 ± 11.1 mg/l) or fourteen days (256.94 ± 17.06 mg/l) (2007). Samples were pressed for 10 min in a pressure-controlled machine at 1 bar (Longarone 85, QS System GmbH, Norderstedt, Germany). Weight and volume of the pressed wines were recorded. The weight of the pomace was recorded and the pomace frozen at -20 °C for further analyses. Wines were transferred into a 100 ml brown bottle and allowed to settle at 4 °C for 24 hours, racked into a 50 ml bottle afterwards and the sediment (yeast lees) was centrifuged (5430R, Eppendorf, Hamburg, Germany) for seven minutes at 6000 g at 20 °C. The resulting pellet was considered as lees, weight recorded and the lees frozen at -20 °C for further analyses. Phenolic analysis was performed according to the Harbertson-Adams assay on samples of the resulting wines in-between one week.

Grape material experimental setup

A rootstock trial (section A) was established in 2003 at Hochschule Geisenheim University in the Rheingau Region (Germany; 49°98'77.9"N 7°93'98.5"E; Blank et al., 2018). *Vitis vinifera* L. cv. 'Pinot Noir', clone Gm1-1 was used as scion and grafted onto five rootstocks: Kober 125AA (125AA), Selection Oppenheim 4 (SO4), Riparia Gloire de Montpellier (Riparia), 110 Richter (R110) and 101-14 Millardet et de Grasset (101-14) in a randomized block design of 14 vines per replicate with four replicates per treatment. Some investigations were carried out on a separated section of the same vineyard (section B) of 'Pinot Noir' grafted on SO4 and used to study the influence of harvest date.

Berry samples for micro-scale winemaking

The first question was to test if the method brought an acceptable repeatability and if its precision changed over ripening and season. For three seasons (2007 to 2009), a selection of four harvest dates separated of a two-weeks period was performed. The first date, 35 days after véraison, unripe around 18 °Brix (30.08.2007; 11.09.2008; 27.08.2009) (DOY 241, 254, 238), the second date, 50 days after véraison as early around 20 °Brix (DOY 260, 267, 252), the third date, 65 days after véraison as standard around 21 °Brix (DOY 276, 277, 267), and the fourth date, 80 days after véraison as late (full ripeness) around 22 °Brix (DOY 288, 293, 280). At each sampling date, 1000 berries of 'Pinot Noir' were selected from the section B of the vineyard and mixed. The berry population was divided in ten subsamples of 100 berries and frozen at -20 °C for a micro-scale winemaking. Fruit from the rootstock trial, section A of the vineyard, was

used for the evaluation of rootstock influence on phenolic concentration in wines. Four replicates per rootstock treatment were selected and 100 berries per replicate frozen at commercial maturity (DOY 264 (2007), 278 (2008) and 266 (2009)).

Influence of freezing

One fundamental aspect was to evaluate the influence of freezing on the extraction of phenolics. 6*100 berries were collected from the section B of the vineyard at commercial harvest (DOY 2007: 289, 2008: 268 and 2009: 267). Three replicates of 100 berries were selected for the treatment "noF" consisting in berries gently crushed, processed fresh without freezing compared to the treatment "F" where berries were frozen at -20 °C for two months, then thawed and crushed.

Larger-scale fermentation

We performed 12 large-scale fermentations (400 kg) with grapes harvested from the section B of the vineyard at the same four harvest dates used for the repeatability test for the three seasons 2007 to 2009. We performed 15 medium-scale fermentations (50 kg) with grapes from the rootstock trial of the section A, harvested equally from the four field replicates of each treatment. A comparison of the two larger-scale fermentation methods was not conducted. Bunches were de-stemmed and crushed and transferred to 40 l (50 kg batches) or 500 l (400 kg batches) fermentation vessels equipped with lids and 50 mg/kg of SO_2 was added. The must was inoculated the next day with 200 mg/l inactive dry yeast Oenoferm® Klosterneuburg (*Saccharomyces cerevisiae*; Erbslöh AG, Geisenheim, Germany). Wines were fermented on skins in a temperature-controlled room at approximately 22 °C for six days and the cap was plunged down three times daily (at approximately 7-hour intervals). Temperature and residual sugar were recorded once a day with a density meter DMA 35N (Anton Paar GmbH, Graz, Austria). The fermentation temperature was between 22 and 25 °C. The pomace was pressed on day 25 with a small membrane press (800 to 1000 kPa). Free-run and press juice were mixed, and a subsample transferred to 25 l glass-balloons equipped with fermentation locks. Wines were racked and bottled six months later in 0.75 l screw-cap bottles.

The Harbertson-Adams assay

The analysis of tannins was performed according to the Harbertson-Adams assay (Harbertson et al., 2002; Harbertson and Spayd, 2006). A protein solution for tannin precipitation was prepared dissolving BSA (Bovine Serum Albumin) in buffer A resulting in a concentration of 1 mg BSA/ml buffer A solution. Skin/seed extracts or wine were diluted in a model wine solution and 1 ml of BSA protein solution was dispensed to react with the tannin. After incubation, the samples were centrifuged (Minispin® Plus, Eppendorf AG, Hamburg, Germany). The supernatant was discarded, 875 µl of the TEA/SDS buffer (containing 5 % TEA (v/v) and 10 % SDS (w/v) adjusted to pH 7.9) was added and the tube was vortexed (Reax-Top, Heidolph Instruments GmbH, Schwabach, Switzerland) to dissolve the pellet. Background absorbance of the solution was red at 510 nm with VIS spectrophotometer (Odyssey, Hach Lange GmbH, Düsseldorf, Germany), and again ten min after the addition of 125 µl ferric chloride reagent (10 mM $FeCl_3$ in 0.01 N HCl). Tannin concentration was calculated from a standard curve as mg CE (Catechin Equivalent). For the analysis of anthocyanins, wine samples were diluted in the model wine solution and 1 ml of maleic buffer was added (Heredia et al., 2006). After five min, absorbance at 520 nm was red. Anthocyanin concentration was calculated as malvidin-3-O-glucoside (M3OG) equivalents.

Statistical analysis

The open source R 3.3.1 statistical computing environment (R Foundation for Statistical Computing, Vienna, Austria) was used for all ANOVA and graphs with the platform Eclipse Mars Release (4.5.2; Eclipse Foundation, Ottawa, ON, Canada) and its plug-in StatET 3.5 (WalWare-Team, Dortmund, Germany). Repeatability was calculated as the relative standard deviation (RSD- %) by dividing the standard deviation of the ten subsamples by its mean. The Shapiro-Wilk test [package stats] was used to check the normality assumption while the Levene test [package car] was used to test the homogeneity of variances assumption of an ANOVA. A two-way analysis of variance (ANOVA) was performed for vintage and rootstock while testing for interactions between vin-

tage and rootstock. Differences between treatment means were compared using the Tukey HSD test (P = 0.01 or P = 0.05).

Table 1: Repeatability of the micro-scale method for total soluble solids (TSS) in juice and for the extraction of phenolics in wines for tannins (T), total iron reactive phenolics (TP) and anthocyanins (Anths); at each sampling date, 1000 berries were mixed and divided in ten subsamples for a micro-scale winemaking fermentation. For each season (2007, 2008, 2009), four dates were selected. A: unripe (~18 °Brix); B: early (~20 °Brix); C: standard (~21 °Brix) and D: late (full ripeness, ~22 °Brix); ± SD; ± RSD %: relative standard deviation

	TSS (°Brix)	RSD %	Wine T (mg/l)	RSD %	Wine TP (mg/l)	RSD %	Wine Anths (mg/l)	RSD %
	Season 2007	**2.07%**		**11.67**		**9.11%**		**7.59%**
A	16.51 ± 0.81^{d}	4.91%	565.19 ± 70.42^{a}	12.46 %	3237.93 ± 263.93^{a}	8.15%	175.49 ± 15.53^{c}	8.85%
B	20.31 ± 0.24^{c}	1.18%	476.59 ± 56.38^{b}	11.84 %	2696.44 ± 241.42^{b}	8.95%	193.54 ± 15.34^{b}	7.93%
C	21.99 ± 0.21^{b}	0.95%	408.18 ± 48.81^{c}	11.96 %	2532.52 ± 205.51^{b}	8.11%	217.52 ± 21.43^{ab}	9.85%
D	22.93 ± 0.28^{a}	1.22%	236.72 ± 24.66^{d}	10.42 %	1762.26 ± 197.53^{c}	11.21%	227.09 ± 8.52^{a}	3.75%
	Season 2008	**1.41%**		**7.76%**		**7.04%**		**11.79**
A	17.95 ± 0.29^{c}	1.62%	678.69 ± 35.16^{a}	5.18%	2748.71 ± 130.86^{a}	4.76%	149.15 ± 30.49^{c}	20.44 %
B	20.16 ± 0.23^{b}	1.14%	607.41 ± 25.52^{b}	4.25%	2689.88 ± 179.02^{a}	6.66%	184.01 ± 21.93^{b}	11.92 %
C	20.55 ± 0.27^{ab}	1.31%	452.42 ± 54.19^{c}	11.98	2262.12 ± 219.88^{b}	9.72%	203.98 ± 23.85^{ab}	11.69 %
D	20.94 ± 0.33^{a}	1.58%	358.17 ± 34.38^{d}	9.63%	2192.5 ± 153.51^{b}	7.02%	234.47 ± 7.27^{a}	3.10%
	Season 2009	**2.52%**		**10.48**		**9.74%**		**17.02**
A	18.35 ± 0.65^{c}	3.54%	949.73 ± 113.8^{a}	11.98 %	3148.12 ± 361.23^{a}	11.47%	74.14 ± 28.41^{c}	38.32 %
B	20.62 ± 0.63^{b}	3.06%	705.39 ± 89.4^{b}	12.67 %	2450.19 ± 322.24^{b}	13.15%	128.86 ± 20.42^{b}	15.83 %
C	20.94 ± 0.38^{b}	1.81%	505.9 ± 51.76^{c}	10.23 %	2043.85 ± 164.01^{c}	8.02%	178.72 ± 10.03^{a}	5.61%
D	22.35 ± 0.37^{a}	1.66%	356.81 ± 25.07^{d}	7.03%	1753.39 ± 111.07^{c}	6.33%	190.28 ± 15.82^{a}	8.31%
	General mean	**2.04%**		**9.97%**		**8.63%**		**12.11 %**

Results

Repeatability of the micro-scale winemaking method over ripening

Repeatability of the micro-scale winemaking method for anthocyanin extraction (Table 1) averaged at 12.1 % when expressed as the relative standard deviation (RSD) considering the whole dataset with an outlier at 38.3 % (2009_A). When discarding the unripe date, RSD for anthocyanins extraction averaged at 8.8 % ranging between 5.6 % and 15.8 %. Wines produced from grapes picked at an unripe date had a significant lower anthocyanin concentration (A: 142.9 mg/l) compared to the other dates (B to D: 183.4 to 195.9 mg/l). Repeatability for anthocyanin extraction into wine depended mainly upon harvest time and was best for later harvested grapes (A: 22.5 %, B: 11.9 %, C: 9.1 % and D: 5.1 %).

Repeatability of the method for extraction of tannins was 9.9 % with a general range between 4.2 % and 12.7 %. Tannin concentration was two times lower in wines produced in 2007 (421.6 mg/l) compared to the other seasons (2008: 524.1 mg/l and 2009: 629.4 mg/l) but the repeatability did not depend on the seasons (2008: 7.7 %, 2009: 10.5 %, 2007: 11.7 %). For wines produced from grapes picked at the unripe date, tannin concentration in wines was high (A: 731.2 mg/l) compared to the early date (B: 596.4) leading to a decrease for the later dates (C: 455.5 and D: 317.2 mg/l). However, the repeatability of the method was independent of the picking date (A: 9.8 %, B: 9.6 %, C: 11.4 % and D: 9.1 %).

Table 2: Repeatability of the analysis of phenolics in wines for tannins, total iron reactive phenolics (TP) and anthocyanins (Anths); at each sampling date, the 10 micro-scale wine replicates were pulled together to obtain a mixture, that analyzed 10 times to test the repeatability of the Harbertson-Adams assay in our lab. For each season (2007, 2008, 2009) 4 harvest dates were selected. A: unripe, around 18 °Brix; B: early, around 20 °Brix; C: standard, around 21 °Brix and D: late (full ripeness) around 22 °Brix; ± SD; ± RSD%: relative standard deviation

	Wine tannins (mg/l)	RSD %	Wine TP (mg/l)	RSD %	Wine Anths (mg/l)	RSD %
Season 2007		**5.60%**		**4.36%**		**2.43%**
A	571.76 ± 35.42	6.19%	3211.17 ± 123.43	3.84%	173.43 ± 6.32	3.64%
B	471.57 ± 24.38	5.17%	2725.91 ± 125.32	4.60%	200.34 ± 4.21	2.10%
C	387.54 ± 21.85	5.64%	2487.03 ± 89.34	3.59%	220.68 ± 3.34	1.51%
D	233.42 ± 12.63	5.41%	1868.46 ± 101.26	5.42%	130.57 ± 3.21	2.46%
Season 2008		**4.23%**		**3.60%**		**2.02%**
A	682.12 ± 25.23	3.70%	2754.57 ± 90.28	3.28%	149.51 ± 4.43	2.96%
B	642.62 ± 26.72	4.16%	2914.42 ± 82.78	2.84%	180.49 ± 3.21	1.78%
C	511.63 ± 24.13	4.72%	2458.89 ± 109.54	4.45%	201.18 ± 2.67	1.33%
D	397.64 ± 17.26	4.34%	2342.07 ± 89.34	3.81%	222.31 ± 4.45	2.00%
Season 2009		**5.56%**		**3.76%**		**3.17%**
A	761.72 ± 59.03	7.75%	2845.67 ± 101.34	3.56%	59.56 ± 1.61	2.70%
B	578.61 ± 27.33	4.72%	1850.48 ± 89.35	4.83%	120.19 ± 3.12	2.60%
C	536.16 ± 31.45	5.87%	2215.14 ± 72.22	3.26%	174.12 ± 5.67	3.26%
D	289.06 ± 11.23	3.89%	1680.77 ± 57.22	3.40%	190.92 ± 7.89	4.13%
General mean		**5.13%**		**3.91%**		**2.54%**

Repeatability of the phenolic analysis

The previous results show that the method described here of micro-scale winemaking of 100 berries is repeatable for fermentation process, extraction and analysis of the tannin components by 4.2 % to 12.7 %. However, not only the winemaking procedure brings variability into the process, but also the method of analysis. A representative average wine was obtained combining the ten replicates for each harvest date. This wine was then analyzed in ten independent replicates to determine the repeatability of the Harberston-Adams assay. The RSD represented in average 5.1 % of the mean tannin concentration (range 3.7 % to 7.7 %) whereas for total iron reactive phenolics (TP), the RSD was lower at 3.9 % (range 2.8 % to 5.4 %; Table 2). Results for total anthocyanins showed that the method of analysis presented a high repeatability as the RSD was 2.2 % of the mean ranging from 1.3 % to 4.1 %.

Influence of freezing

Our investigations showed that freezing the berries led to an increased extraction around 20 % for tannins and 35 % for anthocyanins (Table 3).

Macro-scale fermentation treatments

The extraction and evolution of phenolics was followed for the macro-scale fermentation on 400 kg batches (8 wines) for the four harvest dates in 2007 and 2008 (Fig. 2) and on 50 kg batches from the rootstock trial (15 wines; data not shown). The concentration of tannins increased as the fermentation was completed, peaked as the wines were pressed and remained quite stable thereafter. For the extraction of anthocyanins, the concentration peaked at day 5 after crushing and from then to day 120 anthocyanins decreased by 39.2 % during bottle aging.

Table 3: Investigation on the influence of freezing on phenolics in wines for tannins, total iron reactive phenolics (TP) and anthocyanins (Anths); for each season (2007, 2008, 2009), 600 berries were collected from the section B of the vineyard at commercial harvest (DOY 2007: 289, 2008: 268 and 2009: 267). Subsamples of 100 berries were built to form 3 replicates per treatment. One treatment consisted in berries gently crushed, processed without freezing (noF). For the other treatment, the berries were frozen at -20 °C for 2 months, then thawed at room temperature and gently crushed (F); ± SD

	Wine tannins (mg/l)	**Wine TP (mg/l)**	**Wine Anths (mg/l)**
Season 2007			
F	287.69 ± 70.78	1952.64 ± 81.77	233.92 ± 25.99[a]
no F	256.94 ± 17.06	1587.44 ± 87.75	165.08 ± 3.63[b]
Season 2008			
F	610.54 ± 47.83	2653.93 ± 209.35	230.82 ± 11.05
no F	559.61 ± 15.75	2361.62 ± 115.84	210.11 ± 44.37
Season 2009			
F	197.09 ± 116.87[a]	1605.29 ± 119.84[a]	296.16 ± 3.56[a]
no F	143.99 ± 28.76[b]	1010.18 ± 122.07[b]	197.68 ± 50.72[b]

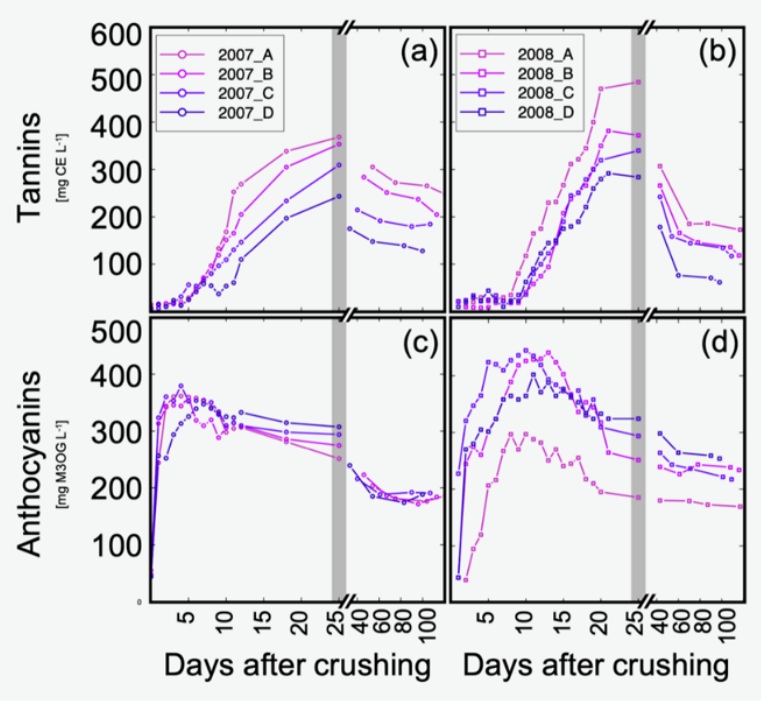

Fig. 2: Extraction and evolution of tannins ((a): 2007 and (b): 2008) and anthocyanins ((c): 2007 and (d): 2008) during maceration and bottle aging of cv. 'Pinot Noir' wines with four different harvest dates (A, B, C, D); CE: catechin equivalents. The gray vertical line indicates the amount in wines at the time of pressing (those values were used for the comparison with micro-scale winemaking as described in Fig. 3).

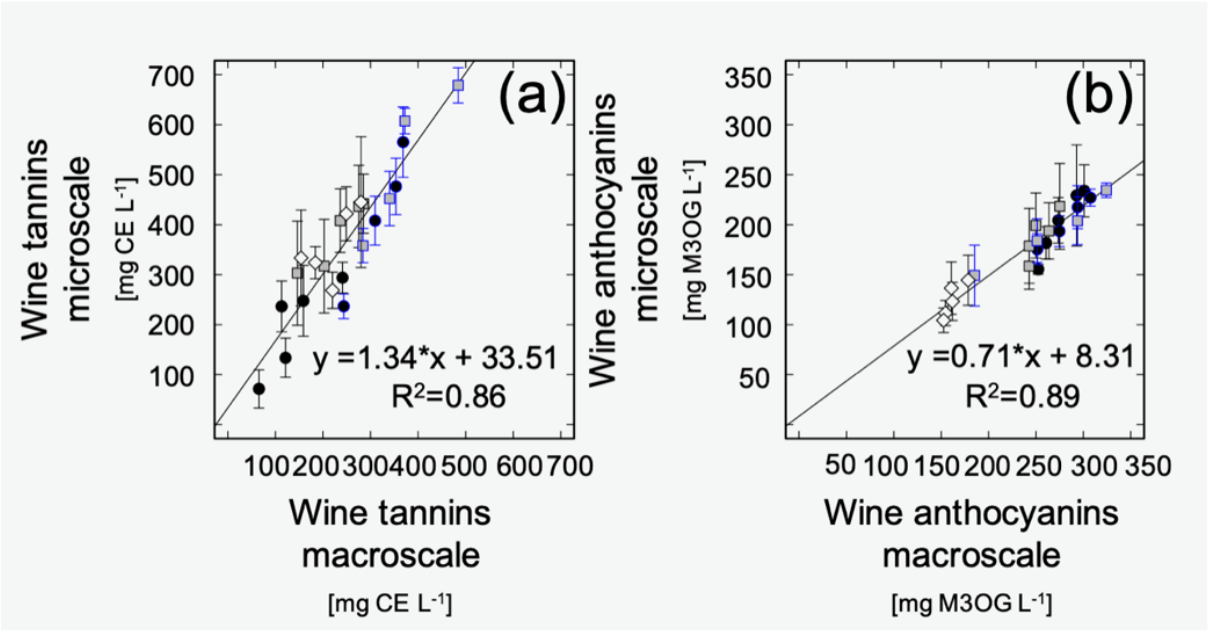

Fig. 3: Correlation between phenolics measured in wines produced by macro-scale compared to those produced by micro-scale fermentation; (a): tannins, (b): anthocyanins; macro-scale fermentations of 50 kg batches of 'Pinot Noir' grafted onto five different rootstocks for 2007 (●), 2008 (□) and 2009 (◇); macro-scale fermentations of 400 kg batches for 'Pinot Noir' at four different harvest dates for 2007 (●) and 2008 (□); concentrations are expressed in mg catechin equivalent (CE) per liter wine for tannins or mg malvidine-3-glucoside per liter wine for anthocyanins

Rootstock influence on phenolic composition in micro-scale wines

As the wines were produced using micro-scale fermentation of each field replicate, a two-way ANOVA on rootstock treatment and year was applied (Table 4). Tannin concentration in wines produced in 2007 was two times lower compared to those produced in 2008 while independently of the seasons, tannin concentration in wines of 'Pinot Noir' grafted on 125AA and Riparia were significantly lower compared to SO4 and R110. This was not explained by yield, as the treatments were similar, nor by vigour expressed as pruning mass, as both extremes in vigour induced to the scion, 125AA and Riparia, led to low tannins in wines. Anthocyanin concentration was slightly higher in wines of 'Pinot Noir' grafted on the low vigor rootstock Riparia.

Correlation between micro-scale wines and macro-scale

The amount of tannin extracted by micro-scale fermentations was related to the amount extracted during larger-scale fermentation (R^2 = 0.86; for 50 kg and 400 kg batches; Figure 3a), arguments for the reliability of the micro-scale method used. The slope of the linear correlation of 1.34, shows a higher extraction by 34 % for micro-scale fermentation in comparison to higher-scale fermentation. For the anthocyanins, the amount extracted by micro-scale fermentations was also related (R^2 = 0.89; Figure 3b) but 29 % lower to the one during macro-scale fermentation.

Table 4: Vigour and phenolic composition of micro-scale wines of 'Pinot Noir' grafted onto five rootstocks (R) for three seasons (Y). The pruning mass and yield were recorded for the 14 vines per replicate. Tannins (T), total iron reactive phenolics (TP) and anthocyanins (Anths) values are the mean (± SD) of four replicates per treatment.

	Pruning mass (T/ha)	**Yield (T/ha)**	**Wine T (mg/l)**	**Wine TP (mg/l)**	**Wine Anths (mg/l)**
Year (Y)					
2007	3.31 ± 0.34^{b}	12.64 ± 3.19^{a}	196.60 ± 93.96^{b}	1282.30 ± 256.57^{b}	200.96 ± 39.38^{a}
2008	3.27 ± 0.57^{b}	8.70 ± 2.52^{b}	381.39 ± 95.35^{a}	1689.01 ± 270.55^{a}	189.74 ± 36.25^{a}
2009	4.03 ± 0.88^{a}	8.72 ± 3.14^{b}	358.57 ± 121.25^{a}	1551.91 ± 399.03^{a}	124.01 ± 29.56^{b}
Rootstock (R)					
Riparia	2.92 ± 0.55^{b}	9.99 ± 3.73	239.77 ± 100.48^{b}	1292.94 ± 275.88^{b}	190.99 ± 51.77
101-14	3.35 ± 0.46^{ab}	10.02 ± 3.63	326.05 ± 111.04^{ab}	1512.58 ± 289.55^{ab}	157.41 ± 41.13
R110	3.44 ± 0.42^{ab}	9.66 ± 3.55	393.65 ± 127.30^{a}	1499.38 ± 327.49^{ab}	162.01 ± 41.15
SO4	3.77 ± 0.54^{a}	11.73 ± 2.78	368.71 ± 113.34^{ab}	1829.30 ± 400.29^{a}	188.08 ± 50.23
125AA	4.12 ± 0.99^{a}	8.96 ± 3.91	232.76 ± 135.04^{b}	1404.48 ± 261.83^{b}	159.36 ± 54.43
Two ways ANOVA					
Year (Y)	*18.62****	13.04***	27.67***	11.28***	37.85***
Rootstock (R)	*8.43****	1.07^{ns}	8.82***	6.33***	3.58*
Y*R	*0.018**	0.46^{ns}	0.52^{ns}	0.54^{ns}	2.31*

*Results of the two-way ANOVA on year (Y) and rootstock (R); main effects and interactions significant at * P<0.05, ** P<0.01, *** P<0.001 or ns (not significant); Values with the same letter within one column are not significantly different at P<0.05 using the Tukey HSD post hoc test.* †

Discussion

A major concern in viticultural research is that measuring grape tannin will not give enough information about the quantity that will be extracted in the corresponding wines (Harbertson et al., 2002; Adams and Scholz, 2007; Busse-Valverde et al., 2011) and makes it hard to conclude to a treatment influence for future wine quality. Several authors attempted to resolve this issue using simulated maceration assays in wine model systems (Gonzalez-Manzano et al., 2004; Canals et al., 2005; Fournand et al., 2006). Though providing interesting results, extraction into a model system presents many drawbacks; principal among these is that the extraction system is not maceration under fermentation conditions (Sampaio et al., 2007). A method for small-scale fermentations was published (Sampaio et al., 2007; Benito et al., 2011) and is known to have low variability and good overall reproducibility (Geffroy et al., 2014). Indeed, variability for tannin analysis across the three replicates was 1.5 to 2.5 % (Sampaio et al., 2007), five replicates was 4.2 % to 18.5 % (calculated from Cerpa-Calderon and Kennedy, 2008) while when considering biological replicates from the vineyard at 13.1 % (Del Castillo et al., 2019). We tested the actual repeatability of our method, using ten replicates of 100 berries at four dates and three seasons. Repeatability of the whole process for anthocyanin extraction in micro-scale wines averaged at 12.3 %, mostly high when considering unripe grapes (12.2 to 22.6 %) compared to the results of mature grapes (5.1 to 8.4 % at full ripeness). Furthermore, we found that repeatability of the whole process for tannins extraction in micro-scale wines averaged at 10.1 % with a general range between 4.1 % and 12.7 %. Thus, we proved the pertinence of the micro-scale winemaking method. The assay used to analyze tannin was the protein binding method adapted from Hagerman and Butler (1978) for grape extracts and wine by Harbertson and Spayd (2006). The method is known to lack in accuracy for both very diluted and concentrated samples (Hagerman and Butler, 1980). We proceeded to the analysis within the valid range in the protein precipitation-tannin assay, where Jensen et al. (2008a) showed that the response minimally suffers from these problems. Indeed, the repeatability of the assay for tannin analysis was around 5 % (range 3.7 % to 7.7 %) in pooled samples, confirming the validity of the Harbertson-Adams assay for tannin

determination used, contradicting the poor precision findings of Brooks et al. (2008).

Most critics against micro-scale winemaking concentrate on the fact that its extraction would not be representative for larger-scale fermentations. Therefore, we made a point to compare the results from micro-scale winemaking with larger-scale (50 kg and 400 kg) fermentations. We found that tannin concentration from micro-scale wines was highly correlated (R^2 = 0.86) with the concentration achieved from larger-scale fermentation of the treatments (n = 23). This presents a striking argument for the micro-scale method used considering that the field replicates (n = 4) were fermented separately, applying a higher variability to the system. It should be noted that our results were obtained with fermentation temperatures maintained under 25 °C, different outcome may be achieved when conducting fermentations with higher temperatures, as an increase in phenolic extraction would be expected with higher temperatures up to 30 °C (Girard et al., 1997 and 2001). We actually found a correlation between tannin concentration in macro-scale wines and astringency evaluated by the panels nine months after bottling. Indeed, tannins determined by Harberston-Adams assay were already correlated with perceived astringency in the wines (Kennedy et al., 2006). The quantity of wines produced by micro-scale winemaking (80 ml) did not allow us to perform a sensory evaluation of the wines but by pulling the replicates together, the two groups could be separated (data not shown).

At the same time, it should be underlined that extraction of total iron reactive phenolics and tannins were higher (by ~8 % and ~34 %, respectively) when berries were fermented by micro-scale winemaking technique. One explanation may be that the berries for micro-scale fermentation were stored at -20 °C prior to fermentation. We found that freezing the berries led to an increased extraction of 20 % for tannins and 35 % for anthocyanins compared to berries processed fresh using this micro-scale winemaking method. In fact, it was shown that freezing the must before fermentation damaged the cell membranes and seems to be an effective technique for releasing both anthocyanins and tannins (Sacchi et al., 2005). When considering the method published (Sampaio et al., 2007), tannin concentration in micro-scale wines (153 mg/l) was ~19 % lower compared to commercial-scale wines (201 mg/l). Their system comprised a submerged-cap system, without agitating the pomace and they observed a lower seed extraction by micro-scale. They suggested that higher temperatures, longer macerations or different cap management would increase the extraction. We implemented a way to punch down the pomace, what could explain the higher extraction of tannins in micro-scale wines (Sacchi et al., 2005). We found that tannin extraction was limited when punching the pomace only once a day compared to more often and set our method at three times.

We found that tannin extraction was not completed after four days post-maceration and it was shown that extending maceration by four days for the micro-scale winemaking technique reduced the differences between commercial and micro-scale winemaking (Sampaio et al., 2007). In fact, tannin concentration increased in wines when maceration time was extended (Sacchi et al., 2005) whereas the percentage of galloylation increased suggesting a higher tannin extraction from the seeds (Gil et al., 2012; Casassa et al., 2013a; Gonzalez-Manzano et al., 2004).

In our study, the larger impact of extending maceration on anthocyanin concentration was shown in wines where a decrease by 40 % was observed when post-fermentation maceration was extended to 16 days. This was also observed for 'Cabernet Sauvignon' and 'Tempranillo' wines as anthocyanin concentration decreased by around 20 % with a longer maceration time of 3 weeks (Gil et al., 2012). Similar results were observed for 'Cabernet Sauvignon' with an extended maceration of 30 days (Casassa et al., 2013b) and 'Merlot' (Casassa et al., 2013a). This may be an explanation for the 31 % lower anthocyanin concentration in micro-scale wines compared to higher-scale fermentations. Anthocyanin concentration in micro-scale wines (363 mg/l), was ~25 % higher compared to commercial-scale wines (291 mg/l; Sampaio et al., 2007), maybe due to a better oxygen management.

We found an increased extraction of anthocyanins in micro-scale wines of 'Pinot Noir' with increasing maturity. Total anthocyanin content also increased throughout ripening in 'Cabernet Sauvignon' and 'Tempranillo' wines (Gil et al., 2012) and other varieties (Perez-Magarino and Gonzalez-SanJose, 2004; Busse-Valverde et al., 2011). Both ripeness and ethanol content have a considerable effect on the extraction of colour (Canals et al., 2005, Fournand et al., 2006). We

found on the other hand, that tannin concentration in wine decreased with increasing maturity. Contradicting results are found in literature on the influence of grape maturity on the extraction of phenolics into wine (Kennedy et al., 2002) and the pattern seems to depend on the season (Pastor del Rio and Kennedy, 2006) but also variety (Gil et al., 2012). The authors showed a higher extraction of seed tannins for lower density grapes (Kontoudakis et al., 2011). Moreover, maturity seems to affect tannin extractability, as it took longer to extract tannins the more mature the grapes were (Gil et al., 2012). Indeed, it seems that while the extractability of skin tannins would be facilitated with maturation (Kennedy et al. 2001), the extractability of tannins would decrease, mostly due to a higher lignification of the seeds (Cadot et al. 2006).

Discussing the influence of the rootstock on 'Pinot Noir' phenolic composition of micro-scale wine is beyond the scope of this paper. It should be, however, noted, that tannin concentration in wines of 'Pinot Noir' grafted on 125AA and Riparia was 40 % lower compared to wines of 'Pinot Noir' grafted on SO4 and R110 for the period covering 2007 to 2009, and similar results were found for 2012 to 2014 (Blank et al., submitted).

Combined with berry analysis (data not shown), it was possible to evaluate the overall extraction rate of grape tannins into wine at an average of 13 %. An incomplete extraction of 20 to 40 % of tannin from grape into wine has already been observed (Jensen et al., 2008b). In our study, when the seasons were considered separately, the relationship between tannin concentration in berries and in wines was stronger in 2009 (R^2 = 0.804; n = 20) compared to 2007 (R^2 = 0.3409; n = 20) and 2008 (R^2 = 0.169; n = 20; data not shown).

Conclusion

We developed a method for micro-scale winemaking to ferment 200 g of berries implementing a way to punch down the pomace allowing a better mechanical cap management and a post-fermentation maceration of two weeks. The first purpose of the experiment was to investigate if the repeatability of the method for phenolic extraction would be suitable. We proved that the micro-scale method described ensured a high repeatability over maturation for different seasons and was at 5.1 to 8.4 % for anthocyanins at full ripeness and 4.1 % to 12.7 % for tannins for 'Pinot Noir'. Most critics of micro-scale winemaking concentrate on the fact that its extraction would not be representative for larger-scale fermentations. The hypothesis that by increasing the post-fermentation maceration period, the micro-scale fermentation method would be comparable to higher-scale fermentation was supported. Indeed, the micro-scale method was validated to larger-scale fermentations, i.e. 50 kg and 400 kg, and that the correlation coefficient was high for tannins (R^2 = 0.86) and anthocyanins (R^2 = 0.89) arguments for the reliability of the micro-scale method used. It should be noted that all the investigations were performed with 'Pinot Noir' samples, results may be different when using other varieties. Different rootstock treatments were used in this trial to induce a maximum in variability with respect to tannin metabolism. Vintage differences were high for overall phenolics levels, but rootstock impact seemed to be beyond weather conditions. Our method made it possible to ferment berries picked from different field replicates separately, respecting the high variability within field trials, and perform a statistical analysis on the results. To conclude, the micro-scale red wine fermentation technique described here showed a high repeatability and indeed is representable for larger-scale fermentation mainly for tannins. Combined with berry analysis, this system could contribute to a better understanding of vineyard management influence on the extraction of phenolic compounds into wines. Ultimately, with this method, it would be possible to investigate phenolic composition in all pre- and post-fermentation products (berries, wines, pomace and lees) as a mass balance approach to finally elucidate the fate of selected phenolics during fermentation.

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