Impact of Leaf Removal, Applied Before and After Flowering, on Anthocyanin, Tannin, and Methoxypyrazine Concentrations in ‘Merlot’ (Vitis vinifera L.) Grapes and Wines

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ABSTRACT: The development and accumulation of secondary metabolites in grapes determine wine color, taste, and aroma. This study aimed to investigate the effect of leaf removal before flowering, a practice recently introduced to reduce cluster compactness and Botrytis rot, on anthocyanin, tannin, and methoxypyrazine concentrations in ‘Merlot’ grapes and wines. Leaf removal before flowering was compared with leaf removal after flowering and an untreated control. No effects on tannin and anthocyanin concentrations in grapes were observed. Both treatments reduced levels of 3-isobutyl-2-methoxypyrazine (IBMP) in the grapes and the derived wines, although the after-flowering treatment did so to a greater degree in the fruit specifically. Leaf removal before flowering can be used to reduce cluster compactness, Botrytis rot, and grape and wine IBMP concentration and to improve wine color intensity but at the expense of cluster weight and vine yield. Leaf removal after flowering accomplishes essentially the same results without loss of yield.

KEYWORDS: antioxidants, aroma, Botrytis, flavonoids, fruit ripening, IBMP, metabolites, polyphenols

INTRODUCTION

Canopy-management practices are used in vineyards to improve cluster microclimate, balance the source—sink relationships, and improve grape composition. The two most utilized practices in commercial settings are cluster thinning and leaf removal. Of these two, leaf removal is arguably the most popular in commercial vineyards. Traditionally, leaf removal is applied in the cluster zone of the canopy between berry set and veraison and generally increases the degree of cluster exposure to sunlight. Cluster exposure can boost or suppress anthocyanin accumulation depending on the maximum temperatures reached by the exposed berries. Moreover, sun-exposed berries can be subject to sunburn, which may negatively impact wine quality. Cluster exposure to sunlight also affects the accumulation and degradation of grape aromatics, specifically of methoxypyrazines (MPs). The berries of several Bordeaux cultivars, such as ‘Merlot’, Cabernet Sauvignon, and ‘Sauvignon blanc’ (V. vinifera L.) can accumulate a significant amount of MPs, key odorants in wines. Sensory notes in the resulting wines are described as bell pepper, asparagus, green pea, or tomato-leaf aromas that, when excessive, can lead to unpleasant vegetative notes, particularly in red wines. Ryona et al. demonstrated that cluster shading led to a greater accumulation of MPs during grape development and at harvest, and several studies have indicated that leaf removal leads to a lower concentration at harvest. Consequently, leaf-

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In viticultural regions characterized by elevated seasonal precipitation, a short growing season, or cool temperatures, grape composition at harvest may be characterized by low anthocyanin levels and high MP concentrations, respectively. Moreover, rainfalls and high humidity during the late stages of fruit ripening can favor the development of Botrytis and other cluster rots,\(^5\)\(^6\)\(^7\)\(^8\) penalizing fruit quality at harvest. In these regions, the application of early leaf removal could be adopted by grape growers as a strategy to (i) improve cluster microclimate favoring increased air circulation and a lower humidity in the cluster zone; (ii) reduce cluster compactness and related chances of Botrytis rot infections; and (iii) increase and reduce the accumulation of anthocyanins and MPs, respectively, thereby improving grape and wine quality.

The objective of this study was to evaluate the effect of leaf removal applied before and after flowering on grape sanitary status, yield, berry secondary metabolites, wine composition, and wine sensory attributes in 'Merlot', one of the most cultivated varieties worldwide. To our knowledge, a simultaneous analysis of anthocyanins, tannins, and MPs during maturation in a red grape variety as affected by leaf removal has never been performed before. Our hypothesis was that leaf removal before flowering could effectively reduce crop size and the incidence of cluster Botrytis rot, possibly favoring sugars and anthocyanins accumulation, reducing MP concentration during berry development and at harvest, and ultimately improving wine sensory features.

**Materials and Methods**

**Chemicals.** 3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-2-methoxypyrazine (IPMP), acetone, methanol (Chromasolv), and perchloric acid were supplied by Sigma-Aldrich (St. Louis, MO). 2-isobutyl-3-methoxy-d3-pyrazine ([2H3]-IBMP) was supplied by C/D/N/Isotopes (Quebec, Canada). Oenin chloride was supplied by Extrasynthese (Genay, France).

**Location, Plant Material, and Experimental Design.** The experimental trial was conducted in a commercial vineyard of the Davino Meroi Winery in the Friuli Grave D.O.C. viticultural area (Pavia di Udine, latitude: 46°00′06″ N; longitude: 13°17′09″ E). 'Merlot' (clone 184, rootstock SO4) grapevines, planted in 2000 at a 2.4 m × 0.8 m spacing (5200 vines per hectare), were used for field experiments in 2012 and 2013. Rows were planted in a north–south orientation, and vines were watered to a single Guyot (10 buds per vine) and trained with a vertical shoot-positioned (VSP) trellis system, with a total canopy height of 90 cm. During the growing seasons, shoots were hedged twice in all treatments: (i) manually when the tips were 30 cm above the catch wire (removed-leaf area measured) and (ii) mechanically (removed-leaf area not measured) on July 13 and July 12, in 2012 and 2013, respectively.

A total of three treatments were set as follows: (i) untreated control (CONT), where all basal leaves were retained in each shoot, (ii) leaf removal before flowering (LRBF), where five to six basal leaves per shoot were removed on May 19 and 20 in 2012 and 2013, respectively, approximately 15 d before flowering (DBF), and (iii) leaf removal after flowering (LRAF), where five to six leaves per shoot were removed on June 23 and 24 in 2012 and 2013, respectively, 15 d after flowering (DAF). Because of the particular behavior of the Guyot training system, the central shoots are shorter, and not always six leaves were unfolded at the time of preflowering leaf removal. When the shoots had less than eight leaves, we removed only five leaves to retain at least one to two small apical leaves per shoot. If present, laterals were retained at both timings of leaf removal. Each treatment was replicated three times in randomly distributed experimental plots of 10 vines each.

The dates of the major phenological stages were assessed. Budburst was recorded on April 10, 2012 and April 18, 2013, flowering (50% cap fall) on June 3, 2012 and June 6, 2013. Veraison (50% red berries) occurred on August 2, 2012 (58 DAF) and on August 7, 2013 (62 DAF). The grapes were harvested when the TSS reached 21°Brix in the CONT on September 22, 2012 (111 DAF) and on September 29, 2013 (114 DAF).

**Leaf-Area Measurements.** Leaf area was assessed on the main and lateral shoots at four different times during the growing season: before and after the application of each leaf-removal treatment, at veraison, and again at harvest. Total leaf area (TLA) and leaf area-to-yield ratio (LA/Y) at harvest were calculated. A sample of 50 leaves of different sizes was collected, and a regression between the main vein length and leaf area was assessed. These measurements were carried out using a leaf-area meter (LI-3100, LI-COR, Lincoln, NE). On each date of measurement, the lengths of the main vein of each of the leaves were measured for one vine per plot, taking care to collect information by individual shoot and to keep main leaves separate from the lateral. With this information, a second correlation between the number of leaves per shoot (separately for main and lateral leaves) and the leaf area was then calculated. Finally, the number of leaves per shoot was counted, again keeping main leaves separate from lateral, in an additional two plants per plot. In summary, leaf area was computed for three vines per plot using the two regression models mentioned above. Total leaf area was calculated by summing the leaf area of the main and lateral shoots. Leaf area-to-yield ratio at harvest was calculated after yield had been determined.

**Flowers and Berries Per Cluster.** A random sample of 10 clusters per plot was collected at the time of LRBF and the number of flowers counted. Similarly, 10 clusters per plot were collected at berry set to determine the number of berries per cluster.

**Yield and Botrytis Rot Estimation.** Yield parameters (cluster weight and cluster number per vine) were collected at harvest for 10 vines per plot. A total of 50 randomly selected clusters from each plot were weighed, and their lengths were measured to calculate an index of grape compactness by dividing the cluster mass by the cluster length.\(^9\)\(^10\) In both seasons, the same 50 clusters were visually inspected for determining the severity of Botrytis infection as described in Sernard Lemut et al.,\(^10\) however, no signs of infection were observed in 2012, and therefore, only 2013 data are presented.

**Berry Sampling and Juice Analysis.** Berries were collected every 12−14 d from approximately 40 DAF until harvest. Samples were harvested and immediately stored in an insulated cooler and transported to the laboratory within 1 h. On each sampling date, one set of 50 and one of 30 berries were sampled from each plot. The first set was collected to measure the TSS, pH, and titratable acidity (TA) of the juice, and the second set was used to measure the concentration of anthocyanins, skin and seed tannins, and MPs. Berries for juice measurement were weighed and manually pressed at room temperature. Total soluble solids (°Brix) and pH were measured using a manual refractometer (ATC-1, Atago, Tokyo, Japan) and a pH meter (HI2211, Hanna Instruments, Woonsocket, RI), respectively. Titratable acidity (expressed as g/L tartaric acid equivalents) was determined by titration of the juice with NaOH 0.1 N until a pH
8.2 The second set of 30 berries was weighed and immediately stored at −80 °C.

Determination of Anthocyanin and Tannin Concentration. Skin and seeds were separated from the frozen berries using a scalpel. After separation, berry tissues were immediately dropped into liquid nitrogen, weighed, and ground to a fine powder with an A11B IKA analytic mill (Königswinter, Germany). An aliquot of 1.8 mL of methanol in water in a 1:1 ratio (v/v) was added to 0.18 g of skin powder in a 2 mL microtube for the anthocyanin extraction. The extraction was performed at room temperature in an ultrasonic bath for 1 h. Samples were then centrifuged at 15 000 rpm for 15 min, diluted, and filtered using regenerated cellulose membranes with a pore size of 0.2 μm (15 mm syringe filter, Phenomenex). Anthocyanin concentration and profile were determined with an HPLC (LC-20AT, Shimadzu) equipped with a diode array detector (SPD-M 20 A, Shimadzu). Separation was performed using a C-18 column (LiChroCART 250−4, Merck) maintained at 25 °C. Solvent A was methanol, and solvent B perchloric acid (0.3%) in water with a flow rate of 0.5 mL/min. The gradient of mobile phase A was as follows: 0−32 min at 27%, 32−45 min at 67.5%, 45−50 min at 100%, and 50−60 min at 27%. Individual anthocyanins were detected at 520 nm and identified by comparing the retention time of each chromatographic peak with available data in the literature.21 The concentration of individual anthocyanins was expressed in oenin chloride equivalents as mg/g of fresh berry.

The analysis of tannins from skins and seeds was performed as described in Herrera et al. Briefly, 0.18 g of skin or seed powder was added to 1.8 mL of a solution of acetone in water, formulated in a 70:30 ratio (v/v), in a 2 mL microtube. Extraction was performed in agitation for 24 h at room temperature. Then the sample was centrifuged, a 1 mL aliquot of supernatant taken, and the acetone evaporated via 1 h of speed vacuum. The residual aqueous extract was adjusted to 1 mL with deionized water. The acetone evaporated via 1 h of speed vacuum. The residual aqueous extract was added to 1.8 mL of a solution of acetone in water, formulated in a 70:30 ratio (v/v), in a 2 mL microtube. Extraction was performed in agitation for 24 h at room temperature. Then the sample was centrifuged, a 1 mL aliquot of supernatant taken, and the acetone evaporated via 1 h of speed vacuum. The residual aqueous extract was adjusted to 1 mL with deionized water. The protein precipitation assay was utilized to measure skin and seed tannins, which were expressed as mg per berry and mg per g of fresh berry.

Determination of Methoxypyrazines. Standards and Solvents Preparation. Standards used included 3-isobutyl-2-methoxyypyrazine (IBMP) with a purity of 99%; 2-isobutyl-3-methoxy-d3-pyrazine ([2H3]-IBMP) with a purity of 99%; and 3-isopropyl-2-methoxyypyrazine (IPMP) with a purity of 99%. Stock solutions of IBMP (250 mg/L), [2H3]-IBMP (500 mg/L), and IPMP (280 mg/L) were prepared in methanol. A working solution of IBMP and IPMP (IBMP = 50 mg/L + IPMP = 56 mg/L) and one of [2H3]-IBMP (0.5 μg/L) were prepared in water purified by a Milli-Q system (Bedford, MA).

Calibration standards were prepared in Milli-Q water purified using working solutions of IBMP, IPMP, and [2H3]-IBMP. A total of 3 g of NaCl were placed into a 20 mL SPME vial along with a stir bar, and 6 mL of Milli-Q water, 2 mL of the working solution of IBMP and IPMP, 2 mL of 4 M NaOH, and 100 μL of the working solution of [2H3]-IBMP were added. The vial was closed and placed onto a magnetic stir plate before the run to dissolve the NaCl.

Sample Preparation and Chromatographic Run. A total of 3 g of NaCl were placed into a 20 mL SPME vial along with a stir bar, followed by 2 g of grape powder, 6 mL of Milli-Q system water, 2 mL of 4 M NaOH, and 100 μL of working solution of [2H3]-IBMP. The vial was closed and placed onto a magnetic stir plate before the run to dissolve the NaCl. The amount of grape tissue to use for the MP analysis was determined by experiment, whereby different aliquots (1, 2, and 3 g of grape powder) were tested. To keep the same head space in the vial, we increased the volume of Milli-Q water reported above by 1 mL when 1 g of grape powder was added and decreased by 1 mL when 3 g of grape sample was added. The volume of NaOH and [2H3]-IBMP and the mass of NaCl were the same as described in the preparation of the sample. Results were compared by paired t test and, as it showed no significant difference among the samples, 2 g of grape powder was used for sample analyses.

The concentration of methoxypyrazines was determined using a gas chromatograph (Agilent Technologies 7890A, Shanghai, China) equipped with a Gerstel MPS2 multipurpose sampler (Gerstel, Mülheim an der Ruhr, Germany) and two serially connected columns, as HP 1 MS (Agilent Technologies, 30 m, 0.32 mm i.d., 0.25 μm film thickness) and an HP INNOWAX (Agilent Technologies, 30 m, 0.32 mm i.d., 0.25 μm film thickness). The extraction was performed on fiber DVB/CAR/PDMS (Supelco, Bellefonte, PA). For quantitative determination, retention time and mass spectrum in selective ion monitoring mode (SIM) were used. The method is described in detail in Šuklj et al.24 Linearity was verified by using calibration standards of different concentration levels (three repetitions for one concentration level, 10 concentration levels for the calibration curve). Linearity and range were determined by multiple linear regressions using the F-test. Calibration curves were derived using increasing amounts of IBMP and IPMP (both 0.8−160 ng/kg) in calibration standards. Good linearity was obtained for both compounds: IBMP (R² = 0.9968) and IPMP (R² = 0.9963). The limit of detection (LD) and the limit of quantitation (LQ) were calculated from the calibration curve. For both IBMP and IPMP, the LD was 0.7 ng/kg. The LQ for IBMP and IPMP was 2.2 and 2.5 ng/kg, respectively. Recoveries were obtained by analyzing spiked samples of grape powder (10 parallel samples per concentration level). The average of the recoveries was calculated. The results are given in Tables S1 and S2. To determine the optimal grape powder mass in the SPME vial, we added different quantities of grape powder to an SPME vial, and their effect on determined content was tested.

Microvinification and Wine Analyses. A total of nine independent microvinifications, one from each experimental plot, were performed as described in Herrera et al. Briefly, 20 kg of grapes from each experimental plot were harvested manually and transported nearby to the experimental winery of the University of Udine, mechanically destemmed and crushed, and transferred to 25 L glass fermentation containers. Musts were fermented at 18 °C for 10 d on the skins and punched down twice daily. After alcoholic fermentation, the wines were pressed and 25 mg/L of SO2 added. Wines were racked twice, at 10 and 30 d after the end of fermentation, and then immediately bottled in 0.5 L bottles closed with synthetic stoppers. Bottles were stored at 10 °C for 4 months until chemical and sensory analyses were performed.

The wine chemical parameters (alcohol, titratable acidity, pH, malic and tartaric acid, and total extracts) were analyzed with a WineScanTM FT120 Basic spectrometer (FOSS, Hillerød, Denmark), and MPs in wine samples were determined as described in Šuklj et al. Wine color intensity (OD420 nm and OD520 nm), color hue (OD420 nm and OD520 nm), and the concentrations of anthocyanins and tannins were determined by spectrophotometry (Uvikon 922, Kontron Instruments). Sensory analyses of the wines were performed as described in Herrera et al. considering the following attributes: color (color intensity and hue), taste (acidity, bitterness, astringency, and minerality), aroma (intensity, fruity, herbaceous, and spicy), and...
Table 1. Canopy and Yield Components in ‘Merlot’ Vines Subjected to Leaf-Removal Treatments in 2012 and 2013  
<table>
<thead>
<tr>
<th>Treatment (T)</th>
<th>Season (S)</th>
<th>Interaction (T × S)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2012</td>
<td>2013</td>
</tr>
<tr>
<td>main shoot LA (m² per vine)</td>
<td>1.32</td>
<td>0.93</td>
</tr>
<tr>
<td>lateral shoot LA (m² per vine)</td>
<td>3.29</td>
<td>3.52</td>
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<tr>
<td>total LA (m² per vine)</td>
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<td>4.35</td>
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<tr>
<td>clusters per vine</td>
<td>10.5</td>
<td>11.1</td>
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<td>flowers per cluster</td>
<td>662.2</td>
<td>599.8</td>
</tr>
<tr>
<td>berries per cluster</td>
<td>154.4 a b</td>
<td>116.7 b</td>
</tr>
<tr>
<td>berry set (%)</td>
<td>23.9</td>
<td>20.9</td>
</tr>
<tr>
<td>cluster weight (g)</td>
<td>215.5 a b</td>
<td>162.3 b</td>
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<tr>
<td>yield (kg per vine)</td>
<td>1.96</td>
<td>1.60</td>
</tr>
<tr>
<td>berry weight (g)</td>
<td>1.68</td>
<td>1.66</td>
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<td>rachis length (cm)</td>
<td>18.2</td>
<td>17.2</td>
</tr>
<tr>
<td>cluster compactness index (g/cm)</td>
<td>11.9 a</td>
<td>9.4 d</td>
</tr>
<tr>
<td>leaf area per yield (m²/kg)</td>
<td>2.49</td>
<td>2.69</td>
</tr>
</tbody>
</table>

“Data were analyzed through two-way mixed model ANOVA (ns, not significant; *, p < 0.05; ***, p < 0.01; ***, p < 0.001), and when differences were significant, the means were separated using Tukey’s HSD test (p < 0.05).” Different letters (a, b) identify significantly different means. CONT, untreated control; LRBF, leaf removal before flowering; LRAF, leaf removal after flowering.

RESULTS

Leaf Area, Yield Components, and Cluster Health. Leaf-area development was affected by the climatic conditions of the two experimental seasons. In 2012, the low rainfall during May slowed canopy development in the first part of the season (from −20 to +20 d after flowering), and in 2013, the abundant rainfall led to faster growth of canopy leaf area early in the season. Prior to the LRBF treatment, the mean leaf area (LA) of the main shoots in the vines were 0.42 and 0.66 m² per vine in 2012 and 2013, respectively, and the application of the treatment reduced this area by 85% and 56% in 2012 and 2013, respectively. Prior to the LRAF treatment, the LA of the main shoots of the vines were 2.02 and 2.99 m² per vine in 2012 and 2013, respectively, and the LRAF reduced this area by 25% and 28% in 2012 and 2013, respectively. Hedging was performed later during the season for a LA reduction for all treatments.

In both years, despite a significant reduction of the leaf area of the main shoots due to the leaf-removal treatments, TLA was only transiently reduced by the LRBF at the time when the treatment was applied and was not affected by LRAF. The TLA was similar among treatments at harvest (Table 1). Surprisingly, the lateral LA accounted for the 71, 79, and 81% of TLA in CONT, LRBF, and LRAF, respectively (Table 1), but by veraison, the canopy was already fully developed, and laterals were not major competitors for photosynthates.

The number of clusters per vine at flowering was similar in all treatments, and the number of berries per cluster was significantly reduced by LRBF (Table 1). Even if the differences between seasons did not prove significant, in 2013 the number of clusters per vine was lower than in 2012, as well as the number of berries per cluster. This condition consequently resulted in a general lower yield in 2013 across treatments (Table 1). As an average of the two seasons, the CONT vines yielded 1.96 kg per vine, and the CONT mean cluster weight was 215.5 g (Table 1). The leaf-removal treatments did not significantly affect the vine yield, even if an 18% yield reduction was observed on average in LRBF vines (Table 1). Cluster weight was significantly lower in 2013 than in 2012 and was significantly reduced by the LRBF treatment in both seasons (Table 1). Finally, berry weight and rachis length were not affected by the treatments, and a reduction of cluster compactness (−22%) was observed in LRBF as compared with CONT and LRAF.

The crop load, expressed as leaf area per yield, was generally lower in 2012 than in 2013 due to the higher yield per vine in 2012, and no differences among treatments were found (Table 1). In 2012, no Botrytis rot was observed on clusters (data not shown). Conversely, in 2013, substantial rainfall occurred near harvest, stimulating the development of Botrytis rot in the clusters of all treatments. It is noteworthy that, in this season, both leaf removals before and after flowering reduced the severity of Botrytis rot significantly compared to the CONT (Figure 1).

Berry Composition. Leaf-removal treatments applied in this study did not affect the TSS, pH, or TA of the berry juice at harvest (Table 2). However, differences among treatments were observed during berry development and particularly at early stages of fruit ripening.

Anthocyanins and Tannins. Leaf removal did not affect either the concentrations of anthocyanins and tannins at harvest.

retronasal (intensity, persistence, fruity, and herbaceous). A scorecard was used to evaluate the experimental wines. The samples were randomized and served to the panel in three consecutive sets in the same day. Panelists could score each attribute in a 1 (low) to 10 (high) intensity scale with the exception of the attribute “color hue,” for which 1 represented red-violet and 10 red-brown.

Statistical Analyses. Line scatters, histograms, and radar charts were constructed using SigmaPlot 13 (Systat Software GmbH, Erkrath, Germany). Software from SAS Institute Inc. (JMP 7.0) was used for statistical analyses. All the data were processed using a two-way mixed-model ANOVA, where the year was considered as a random factor and the leaf-removal treatment as a fixed factor. When differences among treatments or years were significant, the means were separated using the posthoc Tukey’s Honest Significant Difference (HSD) test (p < 0.05). In the case of significant interaction between the leaf-removal treatment and the year, data were analyzed within each year using a one-way ANOVA test. For the statistical analyses of wine sensory results, the different attributes were subjected to a mixed-model ANOVA with treatments as fixed effects and the panelists and year as random factors. To test the difference between treatments in the Botrytis bunch rot severity, we subjected data to arcsine transformation and to a one-way ANOVA.
Les signification of each anthocyanin accumulated in the berry (Table 3). Both the total anthocyanin concentration and the malvidin-3-glucoside concentration, the major anthocyanin accumulated in ‘Merlot’, were significantly higher in 2012 than in 2013 (Table 2). In 2012, the concentration of anthocyanins increased rapidly from 54 DAF to 95 DAF and then slowed, increasing slightly until harvest (111 DAF) (Figure 2A). In 2013, the trend of anthocyanin accumulation was similar to that of 2012; however, maximum levels of anthocyanins were observed at 102 DAF, 9 days before harvest (Figure 2B). Leaf-removal treatments did not affect anthocyanin accumulation during berry development except at 54 DAA in 2012, when anthocyanin concentration was higher in LRBF than in both CONT and LRAF (Figure 2A). Skin tannins were generally lower in 2013 than in 2012 (Figure 2C,D and Table 2), and the skin tannin content expressed on a per berry basis was, likewise, lower in 2013 than in 2012 (Table 2). Although the concentration of skin tannins decreased with berry development in both seasons (Figure 2C,D), the leaf-removal treatments did not alter the concentration of skin and seed tannins in comparison to the CONT.

The pattern of accumulation of seed tannins was similar to that of skin tannins (Figure 2E,F) with seed-tannin concentration at harvest lower in 2013 than in 2012 (Table 2). Methoxypyrazines. Concentration of IPMP in the berry was below the LOQ for all treatments during the late stages of development and at harvest; therefore, these data are not reported. The major MP detected in the berries was IBMP. A dramatic reduction of IBMP concentration was observed from veraison to harvest in both seasons for all treatments. Both leaf-removal treatments delivered a significant decrease in IBMP concentration at different stages of fruit development (Figure 3A,B). However, at harvest, the concentration of IBMP was significantly lower only in LRAF, while no differences were ascertained for LRBF (Table 2).

Wine Analysis and Sensory Evaluation. For most of the compositional parameters analyzed on the finished wines, no differences among treatments were revealed (Table 4). However, a significant interaction year × treatment was measured for the concentration of IBMP in the derived wines, and therefore, a one-way ANOVA was performed within each season. From this analysis, the CONT wines had the highest IBMP concentration in both seasons (Figure 4B). Interestingly, in 2012, LRBF wines had a lower concentration of IBMP than LRAF wines, and in 2013, no differences were observed.

The concentration of anthocyanins in the wines was not significantly affected by the treatments, while in case of tannins a significant treatment and season interaction was observed (Table 4). The comparison of the treatments within each season revealed that the concentration of tannins in wines was significantly higher in LRBF than in CONT in both seasons and higher in LRAF than in CONT only in 2013 (Figure 4B).

The sensory analyses of the wines revealed few differences between treatments (Figure 5). Among sensory attributes, only astrinngency was perceived significantly higher in LRAF than in LRBF and CONT. Finally, despite the lack of differences in anthocyanin concentrations and profiles, color intensity was judged to be significantly higher in LRBF and LRAF wines than in those from the CONT. Moreover, differences among seasons were tested significant for most of the sensory traits analyzed, and no interactions between treatment and season were observed for any of the parameters tested.

**DISCUSSION**

Leaf removal applied before flowering reduced cluster compactness, cluster weight, and Botrytis rot on the clusters at harvest. When applied after flowering, the leaf-removal treatment successfully reduced Botrytis rot severity while not affecting yield components significantly. Leaf removal before flowering reduced fruit set and, hence, the number of berries per cluster. Similar experiments carried out on Tempranillo, 16, 18 Carignano and Carignan, 27 and in Pinot noir, 19 in which leaves were removed ca. 10 days before flowering, indicated that the reduction in the number of berries per cluster also translates into lower yield. In our study, a significant reduction of yield was not observed when the statistical analysis considered the year as a random factor. Indeed, examining the data within each season, a significant treatment × season interaction was observed (Table 4).

### Table 2. Grape Composition in ‘Merlot’ Vines Subjected to Leaf-Removal Treatments in 2012 and 2013

<table>
<thead>
<tr>
<th>treatment (T)</th>
<th>CONT</th>
<th>LRBF</th>
<th>LRAF</th>
<th>season (S)</th>
<th>interaction (T × S)</th>
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<tr>
<td>total soluble solids (°Brix)</td>
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<td>titratable acidity</td>
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<tr>
<td>total anthocyanins (mg/g berry)</td>
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<td>1.09</td>
<td>1.09</td>
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<td>ns</td>
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<tr>
<td>skin tannins (mg/g berry)</td>
<td>1.7</td>
<td>1.7</td>
<td>1.7</td>
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<td>ns</td>
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<tr>
<td>seed tannins (mg/g berry)</td>
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<td>2.67</td>
<td>2.67</td>
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<td>ns</td>
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<tr>
<td>IBMP (pg/g berry)</td>
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<td>4.76</td>
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*Data were analyzed through two-way mixed model ANOVA (ns, not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001), and when differences were significant, the means were separated using Tukey’s HSD test (p < 0.05). Different letters (a, b) identify significantly different means. CONT, untreated control; LRBF, leaf removal before flowering; LRAF, leaf removal after flowering.*
significant reduction of the yield in LRBF vines was observed in 2012 but not in 2013. The lack of significance in the latter season was due to the severity of Botrytis rot on CONT clusters. Indeed, higher rainfalls characterized 2013 during the last stages of ripening (September) and along with diffused Botrytis rot in the vineyard. In this season, leaf removal before flowering effectively improved cluster health (Figure 1). Rotten berries normally lose part of their water content and, as a consequence, the berry weight decreases. The control and LRAF clusters had a relatively higher number of rotten berries compared to LRBF, and this most likely determined the reduction of cluster weight and yield in CONT and LRAF vines, as well as the lack of significance in the vine yield between LRBF and the other two treatments.

Despite the fact that LRBF did reduce cluster compactness, the severity of Botrytis in this treatment was similar than the one observed in LRAF, even though the latter treatment did not modify cluster architecture. This indicates that, in a variety characterized by a relatively loose cluster, such as ‘Merlot’, leaf-removal treatments reduce the severity of Botrytis, mostly by improving the cluster microclimate and by favoring the pesticide penetration.

Neither of the leaf-removal treatments affected TSS and TA at harvest; however, the LRBF treatment resulted in higher TA at early stages of fruit ripening in both seasons and lower TSS at 78...
Table 4. Composition of 'Merlot' Wine Produced from Grapes of Vines Subjected to Leaf-Removal Treatments in 2012 and 2013

<table>
<thead>
<tr>
<th></th>
<th>treatment (T)</th>
<th>season (S)</th>
<th>interaction (T × S)</th>
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<tbody>
<tr>
<td></td>
<td>CONT</td>
<td>LRBF</td>
<td>LRAF</td>
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<tr>
<td>alcohol (g/L)</td>
<td>12.4</td>
<td>11.9</td>
<td>12.4</td>
</tr>
<tr>
<td>Titratable acidity (g/L)</td>
<td>7.03</td>
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<td>7.20</td>
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<tr>
<td>pH</td>
<td>3.28</td>
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<td>3.24</td>
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<tr>
<td>Malic acid (g/L)</td>
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<td>1.07</td>
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<tr>
<td>Tartaric acid (g/L)</td>
<td>3.03</td>
<td>3.27</td>
<td>3.30</td>
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<tr>
<td>Tannins (mg/L)</td>
<td>262.88</td>
<td>376.12</td>
<td>341.40</td>
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<tr>
<td>Anthocyanins (mg/L)</td>
<td>192.09</td>
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<tr>
<td>IBMP (ng/L)</td>
<td>3.53</td>
<td>2.42</td>
<td>2.45</td>
</tr>
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</table>

"Data were analyzed through two-way mixed model ANOVA (ns, not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001), and when differences were significant, the means were separated using Tukey HSD test (p < 0.05). Different letters (a, b) identify significantly different means. CONT, untreated control; LRBF, leaf removal before flowering; LRAF, leaf removal after flowering.

Figure 4. Concentration of tannin (A) and IBMP (B) in the wines produced from grapes of ‘Merlot’ vines subjected to different leaf-removal treatment in 2012 and 2013. Within each year, the means were separated with Tukey’s HSD test (p < 0.05). Different letters identify significantly different means.

Figure 5. Sensory characteristics of ‘Merlot’ wines obtained from grapes collected on vines subjected to different leaf-removal treatments. Data were processed through two-ways mixed-model ANOVA (ns, not significant; *, p < 0.05; ● CONT, untreated control; ▼ LRBF, leaf removal before flowering; ▲ LRAF, leaf removal after flowering). Differences between treatments are reported in Table S3."

DAA in 2013. The documented effects of leaf removal before flowering on TSS and TA evolution in the berry are controversial. Although an increase of TSS under leaf removal before flowering was observed on ‘Sangiovese’, ‘Trebbiano’, and ‘Tempranillo’, other studies have indicated no effects suggesting that the leaf area-to-yield ratio, the cultivar, or the climate conditions may modulate the effects of these treatments. Similar to TSS, the impact of leaf removal before or after flowering on TA is still controversial. Experiments carried out on ‘Pinot noir’, ‘Trebbiano’, ‘Merlot’ (V. vinifera L.), ‘Cabernet Sauvignon’, and ‘Sangiovese’ reported no impact of this technique on TA. While an increase of TA was shown under leaf removal before flowering in ‘Sangiovese’ and in ‘Tempranillo’, On the basis of the data reported in the experiments mentioned above, no significant difference was found between treatments when the leaf area-to-yield ratio was higher than 1 m²/kg, similar to the levels observed in our experiment (Table 1).

The concentrations of anthocyanins and tannins were nearly unaffected by leaf-removal treatments. Even if solar radiation was not directly measured, the clusters from the leaf-removal treatments were visibly more exposed to sunlight during the summer (visual assessment). Light is a pivotal factor for the biosynthesis of anthocyanins, and it is well-known that cluster exposure to sunlight does not affect tannins in the same way. However, detailed studies on the impact of light on anthocyanin production have shown that the effect of light exposure is not consistent across seasons. Previous studies have demonstrated a positive impact of leaf removal imposed before or after flowering on anthocyanin and phenolic accumulation in the berry showing an uncoupling of anthocyanin biosynthesis, assessed via expression analysis of flavonoid genes, with other primary and secondary metabolic pathways. This anthocyanin increase is thought to be caused by a better cluster microclimate, higher solar radiation on the clusters, and, in the case of leaf removal before flowering, by the reduction of yield and by an increased relative skin mass. According to Kliewer and Dokoozlian, the leaf area-to-yield ratio required for the maximum level of TSS and berry coloration at harvest ranged from 0.8 to 1.2 m²/kg. In our experiment, the LA/Y ratio was above the optimum range in all treatments, suggesting no physiological limitation for the vines. Moreover, our previous
study in ‘Merlot’ indicated that even significant reductions of this ratio (from 1.5 to 1.0 m²/kg) do not have any significant effect on anthocyanin accumulation in the berry, suggesting a lack of relationship between the LA/Y ratio and anthocyanin accumulation in this variety.

Besides the total amount of anthocyanin, environmental cues, and viticultural practices can also affect the anthocyanin profile, i.e., the relative abundance of the different anthocyanins. In this study, the anthocyanin profile was not modified by any of the leaf-removal treatments. The relative abundance of di- and tri- and OH- and OCH₃-substituted anthocyanins was unaffected by leaf removal when applied before flowering (Table 3), in contrast to previous studies. As for tannin content, our data support the results of previous studies indicating that viticultural practices have a limited effect on skin and seed tannin content.

Both leaf-removal treatments affected the concentration of MP’s during berry development; at harvest, significantly lower values were measured in LRAF berries (Table 2). The MP concentration in the berry can be affected by multiple factors, specifically light and temperature, crop level, vigor, and high leaf area-to-yield ratio. Remarkably, leaf-removal treatments have been proven effective for reducing the concentration, particularly in cool climates. Roujou de Boube and Marais et al. reported that leaf removal applied before veraison resulted in a 68% and a 50% reduction of the concentration of IBMP at harvest, respectively. Scheiner et al. highlighted that both the timing and the intensity of leaf removal affect the final concentration of IBMP in grapes. Earlier application of leaf removal (10 DAF) resulted in a greater reduction than later ones (60 DAF), and a high intensity of treatment (first five leaves removed) is more effective than a low intensity (three leaves removed) in reducing the concentration of these compounds at harvest. In accordance with previous studies, in our study the IBMP concentration in the wines produced from vines subjected to leaf removal had a significantly lower concentration of IBMP. However, these differences in concentration did not affect the sensory features of the wines.

A higher concentration of tannins was observed in wines produced from LRB and LRAF grapes in 2013 and from LRAF grapes in 2012 in comparison to the CONT. These results are consistent with previous findings reporting that sunlight cluster exposure improve tannin extractability and results in higher tannin concentrations in wines. Differences in tannin composition could have played a role in determining the slightly higher astringency of LRAF wines. Similarly, copigmentation of anthocyanin with flavonoid and nonflavonoid compounds, among others, could have promoted a slightly higher color intensity in LRBF wines. As is known, flavonols are major copigments in red wines. Although we did not investigate flavonols in this study’s grapes and wines, a higher concentration is normally observed in grapes exposed to sunlight, and thus, it is likely that leaf-removal treatments favored a higher concentration of these compounds in the wines and, consequently, a higher presence of flavonol–anthocyanin copigments.

A significant reduction in the concentration of MP’s in the LRAF berries was observed at harvest and in the derived wines. In conclusion, our data indicate that leaf removal before flowering can be used as an effective strategy to reduce cluster compactness and Botrytis rot, to reduce the concentration of methoxypyrazines in grape and wine, and to improve wine color intensity but at the cost of a reduction in cluster weight and wine yield. Although this approach can be used as an alternative to manual cluster thinning to reduce yield and cluster weight in ‘Merlot’ (V. vinifera L.) grapes, the results of this study indicate that when the leaf area-to-yield ratio is not limiting sugar accumulation, this reduction in yield determined by LRB could be associated with major benefits for grape and wine quality.

These results do not exclude that under higher crop-load levels, LRB could be a valuable tool for improving fruit ripening and composition in ‘Merlot’. Finally, leaf removal applied after flowering also improved cluster health, lowering incidence of Botrytis, and decreased IBMP without affecting yield and cluster weight. However, the differences observed in wine composition suggest that yield reduction via LRB could be profitable for an improvement in wine quality, even in wines with moderate crops sizes.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b01013.

Table S1: recoveries of the method for the determination of IBMP in grapes. Table S2: recoveries of the method for the determination of IPMP in grapes. Table S3: evolution of main, lateral, and total leaf area of Merlot vines exposed to leaf-removal treatments. Table S4: sensory features of ‘Merlot’ wines produced from grapes of vines subjected to leaf removal in 2012 and 2013. Figure S1: monthly distribution of rainfall (A), mean temperature (B), and solar radiation (C) during the seasons 2012 and 2013 in comparison with the historical average 1991–2013. Figure S2: evolution of soluble solids, titratable acidity, and of pH in Merlot grapes exposed to leaf-removal treatments in 2012 and 2013. (PDF)

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**Notes**

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### REFERENCES


