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Microclimate Influence on Mineral and Metabolic Profiles of Grape Berries

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The grape berry microclimate is known to influence berry quality. The effects of the light exposure of grape berry clusters on the composition of berry tissues were studied on the "Merlot" variety grown in a vineyard in Bordeaux, France. The light exposure of the fruiting zone was modified using different intensities of leaf removal, cluster position relative to azimuth, and berry position in the cluster. Light exposures were identified and classified by in situ measurements of berry temperatures. Berries were sampled at maturity (>19 Brix) for determination of skin and/or pulp chemical and metabolic profiles based on (1) chemical and physicochemical measurement of minerals (N, P, K, Ca, Mg), (2) untargeted ^{1}H NMR metabolic fingerprints, and HPLC targeted analyses of (3) amino acids and (4) phenolics. Each profile defined by partial least-square discriminant analysis allowed us to discriminate berries from different light exposure. Discriminant compounds between shaded and light-exposed berries were quercetin-3-glucoside, kaempferol-3-glucoside, myricetin-3-glucoside, and isorhamnetin-3-glucoside for the phenolics, histidine, valine, GABA, alanine, and arginine for the amino acids, and malate for the organic acids. Capacities of the different profiling techniques to discriminate berries were compared. Although the proportion of explained variance from the ^{1}H NMR fingerprint was lower compared to that of chemical measurements, NMR spectroscopy allowed us to identify lit and shaded berries. Light exposure of berries increased the skin and pulp flavonols, histidine and valine contents, and reduced the organic acids, GABA, and alanine contents. All the targeted and nontargeted analytical data sets used made it possible to discriminate sun-exposed and shaded berries. The skin phenolics pattern was the most discriminating and allowed us to sort sun from shade berries. These metabolite classes can be used to qualify berries collected in an undetermined environment. The physiological significance of light and temperature effects on berry composition is discussed.

KEYWORDS: Amino acids; flavonols; ^{1}H NMR; HPLC; metabolic profiles; microclimate; partial least-square; temperature; *Vitis vinifera*

INTRODUCTION

In a vineyard the environment is variable due to the natural soil heterogeneity and the uneven light distribution in the canopy. The amount and the distribution of light intercepted by the vines are determined by the architecture of the vineyard, mainly row orientation, height, width, porosity of the canopy, and distance between rows (*1*). The amount of intercepted light affects the whole plant photosynthetic capacity, water balance, and carbon partitioning between vegetative and reproductive growth (*2*). The source to sink balance is an important parameter that controls berry sugar, organic acids, and secondary metabolites content with qualitative enological potential (*3*−*6*). However, light irradiation of the clusters affects berry composition in many aspects. Berry temperature is increased above air temperature (*7*), and a photochemical effect of the light wavelengths on berry metabolism may be expected. In field experiments it is not easy to separate temperature from photochemical effects (*8*). Net solar radiation absorbed by fruits is mainly converted into heat (*7*). A lower organic acid content (malic acid) is generally reported when the berry temperature is increased in relation to respiration stimulation (*9*). If berry temperature is too high (>40 °C) a decrease in sugar and anthocyanin content is observed (*10*).

Red, blue, and UV photoreceptors can activate key enzymes of the flavonoid pathway such as phenylalanine ammonia lyase, chalcone synthase, and stilbene synthase (*11*, *12*). Phytochromes, cryptochromes, and phototropin are ubiquitous photoreceptors

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that occur in fruits (*13*, *14*). Detailed studies on grape berries are lacking, but it has been suggested that phenolic metabolism can be controlled by light quality (*5*, *15*). Conversely, sun-exposed berries show a high content of flavonols in skin (*10*). This flavonol increase is not due to a temperature effect but to a photochemical one (*8*). Inside the canopy, sun light is screened by the leaves and the red−far-red ratio is lower in the cluster zone (*16*). The amount of UV received by the clusters depends on the fraction of sky view by the cluster (*17*). The main factor of variation of UV irradiation is the local amount of incident UVB, the height of the canopy, and local leaf shading. However, there is a significant correlation between global irradiation and the amount of photoactive radiation in the canopy (*16*). In summary, berry composition is greatly influenced by carbon availability (source−sink balance), berry temperature, and photochemical effects of red and UV radiations. After veraison and during maturation the sugar, flavonol, and anthocyanin contents increase rapidly (*18*) and are changed by light exposure (*19*). Berry light exposure within the fruiting zone is highly variable in time and position. This is due to the variability of the climate, the daily course of the sun, and the local position of the berries (azimuth, shading berries by the leaves or the cluster itself). As the main effect of light is to increase temperature, various locations in the canopy can be classified according to the main daily berry temperature (*20*).

Complex chemical composition differences between living tissues can be assessed by drawing a metabolic profile with chemometric techniques. It has been used successfully in food science (*21*) and plant biology (*22*). The metabolic profiles are defined by multivariate statistical methods (*23*). The analytical techniques currently used for metabolic profiling are HPLC, GC−mass spectrometry, and ^{1}H NMR (*24*−*26*). Mass spectrometry has a higher potential of identification, compared to that of ^{1}H NMR which is limited by low sensitivity, overlapping signals, and dynamic range problems in a crude extract. However, ^{1}H NMR spectroscopy is a promising nontargeted profiling method, because it detects a broad range of metabolites (amino acids, organic acids, sugars, and phenolics) in a quantitative way (*27*). Therefore, ^{1}H NMR can be used for fingerprinting by using the primary data of the spectrum (standard spectral domain). The identification of the significant metabolites is always possible afterward (*26*, *28*).

In this work metabolic profiles of field grown berries were determined based on classical analytical data and ^{1}H NMR spectral pattern recognition (*29*). Partial least-squares (PLS) and linear discriminant analysis were used to compare the capacities of the different profiling techniques to discriminate among exposed and shaded berries and to identify the most significant analytical variables typical of the light environment of the berries.

MATERIALS AND METHODS

Sample Origin. The grape berry samples were harvested in September 2002 at maturity defined as mean °Brix > 20 and titratable acidity (TA) < 100 meq according to the local general commercial practices. Each berry sample, made of 20 berries, was collected in the vineyard and rapidly transferred to the laboratory in an isothermal box at 8 °C.

The vineyard was located close to Bordeaux (France, latitude 44.883°; longitude −0.567°). The grapevines, cultivar "Merlot" were 12 years old, grafted on "Fercal" rootstock (*Vitis berlandieri* × *Vitis vinifera*), and cultivated in a loamy sand soil. The plantation density was 6600 plants/ha (1.5 m between rows, 1 m between vines), north−south row orientation, 1 m canopy height. Shoot trimming was achieved after flowering to maintain the canopy height and width to 1 and 0.3 m, respectively. All other practices were carried out according to standard commercial practice.

Experimental Design. Illumination in the cluster zone depends on the side of the row (east or west), the local leaf density, and the position of the berries in the cluster (outside or inside the canopy). Illumination level was modified using cluster position, the position of the berries in the clusters, and leaf removal treatments. When applied, the leaf removal treatments were implemented in one side only of each vine, either the east-looking or west-looking side, bearing four and one-half bunches on average. They were applied individually to clusters, at veraison (start of berry maturation).

Tree levels of leaf removal, in the fruiting zone, were applied: no leaf removal, 50% leaf removal, and 100% leaf removal (the current practice of commercial growers), 25 cm around the clusters. Due to the course of the sun, the light exposure of the berries is not constant and simultaneous in the different locations. The classification of the berries into shaded or sun-exposed position was based on the daily record of temperature of 18 berries corresponding to 18 different locations: two row sides, three leaf removal treatments (0%, 50%, 100% in the cluster zone), and three positions in the cluster (sun-exposed, middle, or shaded part). The temperature of the berries was recorded with thin thermocouples (*8*). Data were stored continuously from veraison to maturity in a data logger (Campbell 10×, Logan U.S.A.). This sampling was defined to take in account the diversity of the light microclimate in a commercial vineyard. Each location was classified as sunny or shaded according to the bimodal distribution of the 18 sums of temperature records. A weather station next to the plot recorded local climate, air temperature, solar radiation, wind speed, and dew point (Simel, Gevrey Chambertin, France).

To apply statistical analysis on chemical and profiling results of skin and pulp extracts, 60 samples of 20 berries were harvested and sorted into two lots according to the mean daily sum of temperature of the location in the canopy: shaded (38 samples) and sun exposed (22 samples). Twenty samples (8 sun exposed and 12 shaded) were set aside to test the model drawn by linear discriminant analysis after PLS discriminant analysis of the primary data set.

Vine leaf area was estimated by measuring the mean area of 72 primary and secondary leaves sampled randomly on 12 vines and counting the number of leaves on primary and 25% of secondary shoots.

Extraction of Skin and Pulp Tissues. Berry fresh weight was measured. Skins and pulps were separated manually on ice, to carry out two separate extractions, and weighed. The skins were ground with a Waring blender during 2 min at ambient temperature followed by extraction with 96% ethanol on ice during 1 h (40 mL of EtOH per 20 skins). The pulp was centrifuged for 5 min at 3000*g*. One aliquot was used for physicochemical measurements carried out on the pulp. The remnant was extracted on ice with 96% ethanol during 15 min (40 mL of EtOH per 20 pulps).

Physicochemical Characterization of Grape Berries. Five minerals were measured in the pulp: total nitrogen (N), total phosphorus (P), potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}). Total nitrogen and phosphorus were measured following digestion using sulfuric acid and hydrogen peroxide (*30*). Total K^+, Ca^{2+}, and Mg^{2+} contents were determined following dilution of the pulp, by an inductively coupled plasma atomic emission spectrometer (Varian Vista, Varian, Mulgrave, Australia). Phosphate was determined with an automated colorimetric method using TRAACS 800 methods (*31*). Total acidity at pH 7 and pH were determined with an automated pH meter and titration with NaOH (0.1 N). Total soluble solids (°Brix) were determined using a hand refractometer with temperature compensation. Total sugars, tartaric acid, and malic acid concentrations were determined with an automated colorimetric method using the autoanalyzer TRAACS 800 (total sugars reaction with 2-9-dimethyl-1-10-phenanthroline, tartaric reaction with ammonium vanadate, and malic reaction with malic enzyme and cofactors), according to Blouin (*32*). Flavonols (OD_{360}) and anthocyanin contents (OD_{520}) were determined on skin extracts with a UV−vis spectrophotometer Cary 1 Bio at 360 and 520 nm, respectively (Varian Inc. Palo Alto, CA). The ethanolic extracts were acidified by adding 5% of water containing 2% v/v HCl (10 N). The optical densities were measured at 360 and 520 nm (*33*, *34*). Anthocyanins (OD_{520}) content

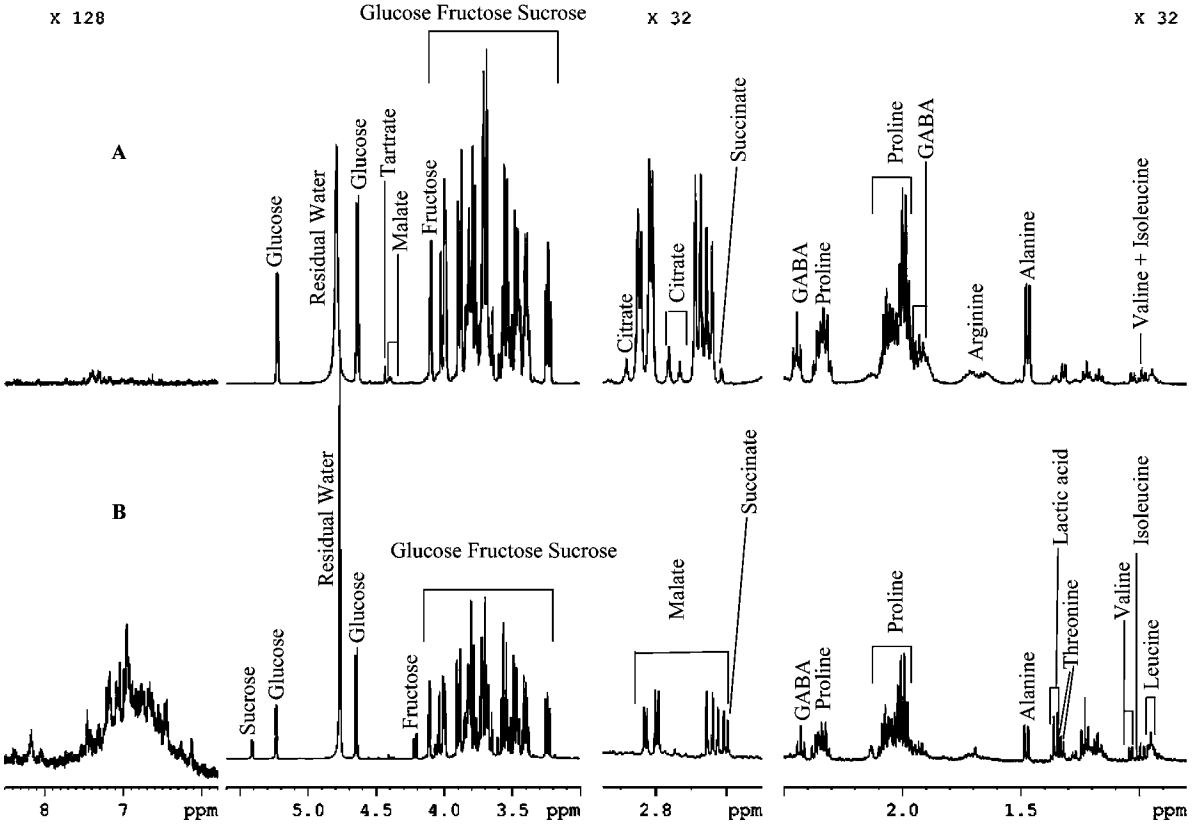

Figure 1. Representative ^{1}H NMR spectra of pulp (**A**) and skin (**B**) extracts of "Merlot" cultivar in shaded condition, with 64 scans and a total acquisition time of 29 min.

was expressed as mg g^{-1} fresh weight (FW) and OD_{360} was expressed as OD_{360} g^{-1} FW.

These measurements were grouped into the "maturity analytical data set" (15 variables) made out of the pulp content of minerals (N, P, K^+, Ca^{2+}, Mg^{2+}), soluble solids, sugars, malic acid, tartaric acid, total acidity, pH, skin anthocyanins and flavonols, mean berry weight, and the skin to pulp fresh weight ratio.

1D ^{1}H NMR Analysis. An aliquot of each skin or pulp extract was dried under vacuum (Speed Vac Savant, Ramsey U.S.A.) then dissolved in 400 mM oxalate buffer, pH 4, and dried again. After concentration under vacuum the residues were dissolved in D_2O, titrated to pH 4.0, stirred during 4 h at 4 °C, and freeze-dried to reduce the residual water signal in the spectra.

The NMR spectra were performed on the titrated extracts dissolved in 0.5 mL of D_2O. The sodium salt of (trimethylsilyl)-propionic-*2,2,3,3-d*$_4$ acid (TMSP) in D_2O was added in all samples at a final concentration of 0.01% for chemical shift calibration at 0 ppm. 1D ^{1}H NMR spectra were recorded at 27 °C with a 500,162 MHz Avance spectrometer (Bruker Biospin, Karlsruhe, Germany) using a 5 mm inverse probe and fitted with an autosampler. Each spectrum consisted in 64 scans of 32 K data points with a spectral width of 6000 Hz, an acquisition time of 2.73 s, and recycle delay of 25 s per scan in order to allow complete relaxation and absolute quantification. The pulse angle was 90°. Spectra were acquired under an automation procedure (automatic shimming and automatic sample loading) requiring about 29 min of acquisition per sample. Preliminary data processing was carried out with XWIN NMR software (Bruker Biospin, Karlsruhe, Germany). Free induction decays (FID) were Fourier transformed with 0.3 Hz line broadening, phased, and baseline corrected using XWIN NMR software (Bruker Biospin, Karlsruhe, Germany). Signal assignment was performed following published data (*28*, *35*) and confirmed with addition of standards.

Quantitative ^{1}H NMR fingerprints were determined on the titrated ethanolic extracts of pulp and skin solubilized in D_2O (**Figure 1**, parts **A** and **B**). The region of the spectrum between 0 and 3 ppm is related to aliphatic compounds, amino acids, and organic acids. The sugar region (3−5.5 ppm) showed a great number of overlapping peaks. The 6−9 pp region points out aromatic compounds, but owing to the complexity of the signals (*36*) ^{1}H NMR does not allow one to identify individual compounds. To use all the information of the spectra signature, each 1D ^{1}H NMR spectrum was baseline corrected and segmented into 190 spectra domains of 0.04 ppm (variables called buckets) using the metabolite mode of AMIX software (Bruker Biospin, Karlsruhe, Germany) between 0.76 and 8.8 ppm (190 variables). The resonances between 4.7 and 5.0 ppm, corresponding to residual water, were removed. All NMR buckets were normalized to the total spectra intensity without the water region. The NMR variables situated between 2.60 and 2.88 ppm (malic, succinic, or citric acids) or 4.36 and 4.48 ppm (tartaric or malic acids) were summed and named S2.8 and S4.4, respectively, to take uncontrolled peak shifts in these organic acid regions into account (*37*). For the other peaks no shift was observed in the spectra.

HPLC Analysis of Free Amino Acids. The amino acid content in skin and pulp extracts was determined according to Cohen and Michaud (*38*). After derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, amino acids were analyzed using an HPLC system consisting in a P100 gradient pump and an AS100 XR automated sampler (Thermo Separation Products, San Jose, CA). Separation was carried out on a Nova-Pack C18 AccQ-Tag (WAT052885, Waters, Milford, U.S.A.) column at 37 °C with elution at 1 mL min^{-1} with a 67 min linear gradient (eluent A, sodium acetate buffer, 140 mM at pH 5.7; eluent B, acetonitrile 60% (v/v) and water). Fluorescence detection was performed with an FL 3000 dual monochromator detector (Thermo Detector Products, San Jose, CA). To maintain consistent retention time and a stable baseline, a control was performed before each run of 18 samples to detect possible trouble in the chromatogram. Peak areas were integrated by Millenium^{32} software, version 3.05 (Waters, Milford, MA). A standard of 20 amino acids (Sigma Aldrich, Lyon, France) was used after the control and in the middle of each run to calibrate the amino acid quantification. Twenty amino acids were identified and quantified in pulp or skin extracts: aspartate, glutamate, asparagine, serine, glycine, glutamine, histidine, threonine, asparagine, alanine, γ-amino-*n*-butyric acid (GABA), proline, tyrosine, cysteine, valine,

Table 1. Means of Berry Weights, Total Sugar, Tartaric and Malic Acid Concentrations, Total Nitrogen, Potassium Amounts, and Flavonols (OD_{360} nm) from Shaded and Sun-Exposed Berries[a]

variables	shaded ($n = 38$)	sun exposed ($n = 22$)
berry weight (g 100 berries^{-1})	212.8 a	203.1 b
percentage of skins (% FW)	8.45 a	8.39 a
total acidity (meq L^{-1})	101.0 a	87.8 b
tartaric acid (meq L^{-1})	96.2 a	90.9 b
malic acid (meq L^{-1})	42.7 a	34.4 b
pH	3.28 b	3.39 a
soluble solids (°Brix)	19.2 b	19.8 a
total sugars (g L^{-1})	197.0 b	211.0 a
total anthocyanins (mg g^{-1} skin)	12.9 a	12.1 a
total flavonols (OD_{360} g^{-1} skin)	58.8 b	80.1 a
total N (μg g^{-1})	541 b	614 a
total P (μg g^{-1})	109 a	112 a
K (μg g^{-1})	2092 a	2032 a
Ca (μg g^{-1})	51.2 a	48.7 a
Mg (μg g^{-1})	69.0 a	64.4 b

[a] In each line, different letters indicate significant differences between means (Bonferroni test, $P < 0.05$). Berry samples were grouped according to the mean sum of temperature above 10 °C of the berries recorded after leaf removal treatment in the 18 different locations combining the three levels of leaf removal, the azimuth of the clusters (east and west), and the position of the berries in the cluster.

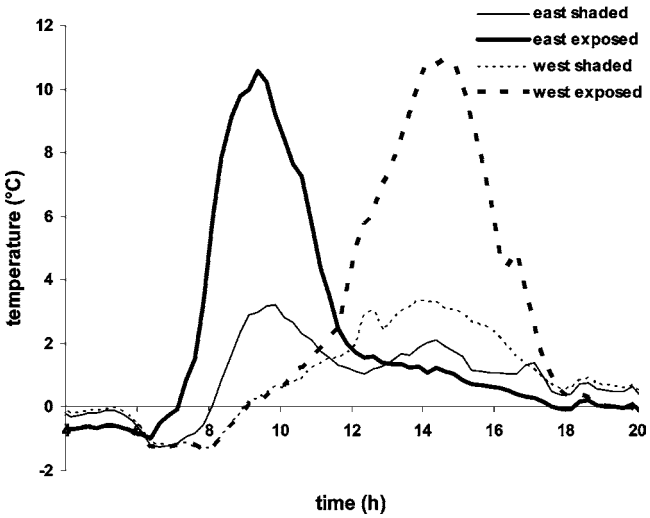

Figure 2. Temperature difference between grape berries and air (°C) along a clear day recorded on sun-exposed and shaded berries on the east and the west side of the canopy. The orientation of the rows was north–south (EO, east shaded; EX, east exposed; WO, west shaded; WX, west exposed).

methionine, isoleucine, leucine, lysine, and phenylalanine. The results were expressed in μg N/100 g fresh weight (FW) of skin or pulp.

HPLC Analysis of Skin and Pulp Phenolic Compounds. A volume of 1 mL of ethanolic extract was dried under vacuum (Speed-Vac, Savant). Then the extract was solubilized in 0.5 mL of acidified methanol at 0.1% of HCl (v/v), filtered through a 0.45 μm membrane (Pall Gelman Corp., Ann Arbor, U.S.A.). A volume of 20 μL of the solution was injected for the HPLC separation (2690 Alliance Separation Module, Waters, Milford, MA) and detected with a UV−V variable wavelength detector operating at 360 and 520 nm (*32*). Separation was achieved on a reversed-phase Ultrasphere ODS column 25 cm × 4.6 mm, 5 μm particle size with an Ultrasphere ODS guard column 4.5 cm × 4.6 mm from Beckman Instruments Inc. (Fullerton, CA), at ambient temperature. Water was purified (18 MΩ) with an ELGA UHQ water purification system (Bucks, U.K.). The duration of one analysis was 80 min, with an elution gradient of solvent A (water and formic acid, 90/10 v/v), and solvent B (water, acetonitrile, and formic acid, 60/30/10 v/v). A binary gradient at 1 mL min^{-1} flow rate was used: 0 min, A 80%, B 20%; 70 min, A 15%, B 85%; 75−80 min, A 80%, B 20%. The column temperature was 25 °C. An external standardization was used for the quantification on the basis of peak area of malvidin-3-glucoside for all the anthocyanins (at 520 nm) and quercetin-3-glucoside as the common standard for all quantified flavonols and unknown compounds (at 360 nm). Malvidin-3-glucoside and quercetin-3-glucoside were purchased from Extrasynthese (Genay, France). Flavonols were analyzed at 360 nm, 20 compounds were quantified, 7 were identified (myricetin-3-glucoside, rutin, quercetin-3-glucoside, myricetin, kaempferol-3-glucoside, isorhamnetin-3-glucoside, and quercetin) and 13 were unknown (X1−X13). Skin anthocyanins (*15*) were detected at 520 nm (delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, paeonidin-3-glucoside, malvidin-3-glucoside, delphinidin-3-(acetyl)-glucoside, cyanidin-3-(acetyl)-glucoside, petunidin-3-(acetyl)-glucoside, paeonidin-3-(acetyl)-glucoside, malvidin-3-(acetyl)-glucoside, delphinidin-3-(*p*-coumaryl)-glucoside, cyanidin-3-(*p*-coumaryl)-glucoside, petunidin-3-(*p*-coumaryl)-glucoside, paeonidin-3-(*p*-coumaryl)-glucoside, and malvidin-3-(*p*-coumaryl)-glucoside. Pulp and skin were characterized by 20 and 35 phenolics compounds, respectively.

Statistical Analyses. All the variables except NMR buckets were analyzed by ANOVA (Systat software). Then significant differences between means were determined with the Bonferoni's test. For multivariate analyses, separated statistical analyses were performed on three groups of variables: group one, berry and skin weight, N, P, K, Ca, Mg, soluble solids, acidity, and phenolics (OD_{520} and OD_{360}); group two, amino acids, anthocyanins, flavonols, in pulp and skin; group three, ^{1}H NMR spectra domains (buckets) in pulp and skin. Partial least-square regression (PLS) multivariate methods were used [Windas software (*23*)]. PLS calculates synthetic variables, and the scores are maximized to discriminate the group of samples according to the studied discriminating factor, here light exposure of the clusters (*29*, *39*).

Chemicals. All the chemical reagents were of analytical grade (Mallinckrodt Baker France, Noisy-Le-Sec, France). D_2O (99.9%) was purchased from Euristop (Gif sur Yvette, France), TSP (98%) from Aldrich (Saint Quentin Fallavier, France). Methanol (HPLC grade) was obtained from Baker (Mallinckrodt Baker France, Noisy-Le-Sec, France) and formic acid (99%) from Merck (Merck Eurolab, Fontenay-sous-Bois, France). Chemical standards for HPLC analyzes were purchased from Sigma (St Louis, MO).

RESULTS

Berry Microclimate. The intensity of leaf removal was measured 1 month before harvest by using the ratio of the leaf area removed divided by the total leaf area. The average leaf area per vine was 2.9 m^2, and the leaf area index was 1.5. Leaf trimming was limited to the fruiting zone. Less than 5% and 10% of the total vine leaf area was removed for the 50% and 100% fruiting zone leaf removal. The mean calculated fraction of incident light intercepted by the vines was the same for the three treatments (59% ± 2).

An example of the daily variations of berry temperature in three conditions, berries exposed on the east and west side and shaded berries, is shown **Figure 2** for a clear day. The integration of the light exposure of the berries in the different positions described in the Material and Methods was quantified by summing the daily berry temperature above 10 °C recorded each 15 min. The summation of the berry temperature was used to separate sample into two classes, sun-exposed and shaded berries.

Discriminant Analysis from Classical Maturity Analytical Data Sets. ANOVA was performed on the 16 variables measured on 60 samples of grape berries, 38 from shaded and 22 from exposed berries (**Table 1**). Differences between sun-exposed and shaded berries were significant for all analytical variables except anthocyanins, P, K, and Ca^{2+} contents. Exposed berries had higher total sugars and flavonols (absorbance at 360

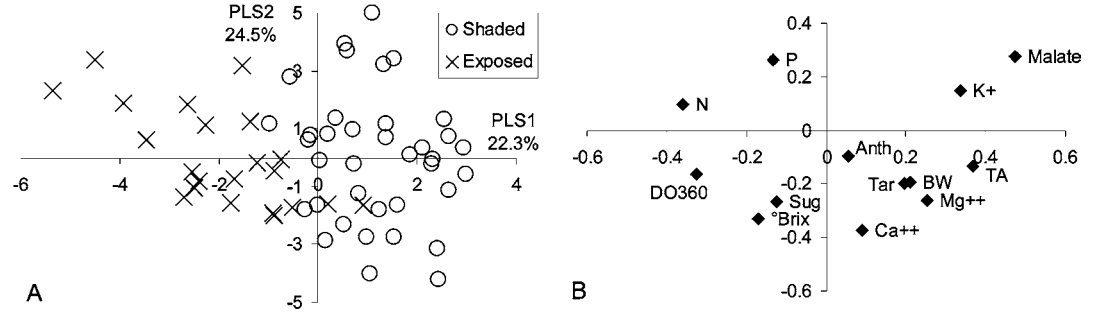

Figure 3. PLS discriminant analysis applied on classical maturity analytical data measured on 22 sun-exposed and 38 shaded berry samples sampled at the mature stage. (**A**) Position of the samples along the first two PLS axes. The percentages of variance explained by each axis are reported. (**B**) Variate loadings for the first two axes (DO360, optical density of the ethanol extract at 360 nm, flavonols; N, total nitrogen; P, total phosphorus; Sug, glucose + fructose; Anth, anthocyanins; Tar, tartrate; BW, mean berry weight; TA, titratable acidity).

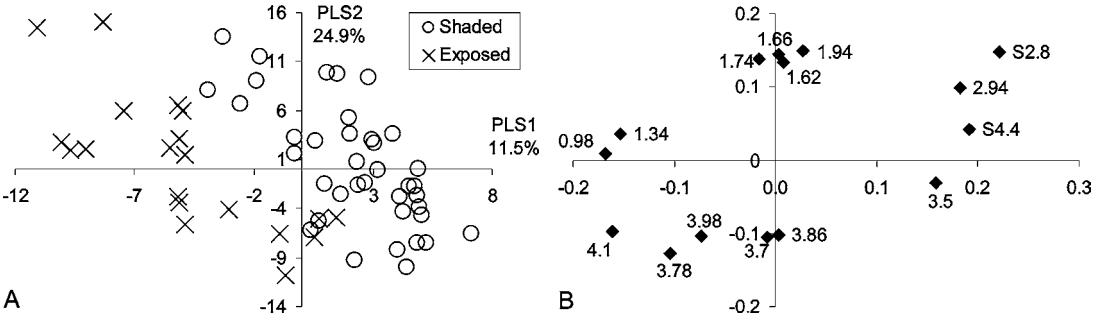

Figure 4. PLS discriminant analysis applied on the 190 buckets extracted from the ^{1}H NMR of the pulp extracts from 22 sun-exposed and 38 shaded berry samples sampled at the mature stage. (**A**) Position of the samples along the first two PLS axes. The percentages of variance explained by the axis are reported. (**B**) Variate loading for the first two axes. Part **B** is the NMR resonance position of the bucket (0.04 ppm) in the spectra. The most significant buckets loading on the first axis are S2.8 and S4.4 (sum of three buckets, see text) representative of protons from malic/citric/succinic acids and malic/tartaric acids, respectively (2.94 ppm, GABA; 0.98 ppm, leucine; 4.1 ppm, sugar; 1.34 ppm, threonine).

nm) and lower total acidity, tartaric, and malic acid concentrations and higher berry weight than shaded berries.

PLS discriminant analysis (**Figure 3A**) was performed on both groups of berries. The first PLS score clearly separated the group of exposed berries from the shaded group. It explained 26% of total variability. This score was loaded by variables related to berry acidity (malate, total acidity, and K^+) on the positive side and N and total flavonols on the negative side (**Figure 3B**). Sugars contributed slightly to the first score. The second PLS score explained 24% of total variability and was related to P on the positive side and Ca^{2+} and sugars on the negative side. It did not contribute to the discrimination of the groups.

Metabolite Profiling by 1D ^{1}H NMR Spectroscopy in Pulp and Skin Berry Extracts. Quantitative ^{1}H NMR fingerprints were determined on the titrated ethanolic extracts of pulp and skin solubilized in D_2O (**Figure 1**, parts **A** and **B**). The region of the spectrum between 0 and 3 ppm is related to aliphatic compounds, amino acids, and organic acids. The sugar region (3−5.5 ppm) showed a great number of overlapping peaks. The 6−9 ppm region pointed out aromatic compounds, but owing to the complexity of the signals (*36*), ^{1}H NMR does not allow one to identify individual compounds.

PLS was applied on the 190 variables issued from the bucketing (0.04 ppm) of each NMR spectra of 60 samples from exposed or shaded berries (**Figure 4**). For pulp extracts, the first two PLS scores explained 36.4% of total variance and allowed us to separate the two groups (**Figure 4A**), except for a few samples. The first PLS score (**Figure 4B**) was loaded by buckets from the aliphatic region (malate, tartrate, citrate, and/or succinate, grouped buckets S2.8 and S4.4) on the positive side. The negative side was loaded by buckets from the aliphatic region (leucine, threonine, 0.98 ppm, 1.34 ppm, respectively) and sugar region (fructose, 4.1 ppm). The second PLS score was described by resonances in the aliphatic region on the positive side (arginine and proline) and sugars on the negative side.

For skin extracts, PLS analysis resulted in a good discrimination between the exposed and shaded groups with the first scores (**Figure 5A**). The first PLS score was loaded by buckets of the aliphatic region of the spectrum (**Figure 5B**), the sugar region and organic acid signals on the positive side (higher amounts in shaded berries). The concerned compounds were malic, and/or citric and/or succinic acids (S2.8 ppm), malic and/or tartaric acids (S4.4 ppm) and sucrose (5.42 ppm) and two unattributed buckets (1.22 and 1.26 ppm). On the negative side, the first PLS score (higher ^{1}H signal amounts for exposed berries) was loaded by buckets of the aliphatic region, valine (1.06 ppm), proline (1.98, 2.34, 2.38 ppm) and in the aromatic domain (6.1 ppm). This first PLS score separated exposed from shaded berries quite well although it explained only 6.2% of total variance. The second PLS score explained 16.2% of total variance and revealed a large intragroup variability. It was loaded by buckets attributed to organic acids (tartaric and/or malic acids, S4.4 ppm, GABA (1.94 ppm) and proline or GABA (1.94 ppm) and arginine (1.66 ppm) on the positive side. On the negative side, it was loaded by buckets in the sugar region (3.54 ppm) and in the aromatic region (7.22 and 8.38 ppm).

Amino Acid Concentrations in Pulp Extracts of Grape Berries. PLS discriminant analysis was applied on the amino acid content of pulp extracts. The separation of the exposed and shaded groups by the first score was satisfactory (**Figure**

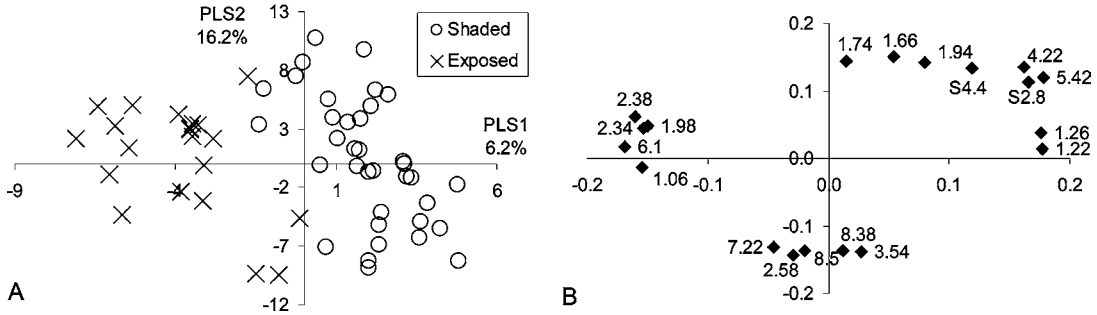

Figure 5. PLS discriminant analysis applied on the 190 buckets extracted from the ^{1}H NMR spectra of the skin extracts of 22 sun-exposed and 38 shaded berry samples sampled at the mature stage. (**A**) Position of the samples along the first two PLS axes. The percentages of variance explained by each axis are reported. (**B**) Variate loadings for the first two axes. Part **B** is the NMR resonance position of the bucket (0.04 ppm) in the spectra. The most significant buckets loading on the first axis are the following: 1.26 and 1.22 ppm, unknown; S2.8 (sum of three buckets, see the Materials and Methods) representative of protons from malic/citric/succinic acids; 5.42 ppm, sucrose; 0.98 ppm, leucine; 4.22 ppm, fructose; 1.98 and 2.34–2.38 ppm, proline; 1.06 ppm, valine; 6.1 ppm, aromatic compound domain.

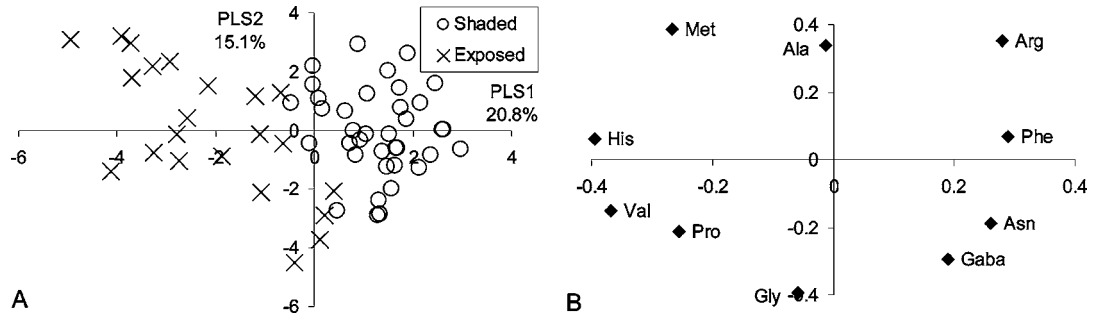

Figure 6. PLS discriminant analysis applied on amino acid analysis of pulp extracts of 22 sun-exposed and 38 shaded berry samples sampled at the mature stage. (**A**) Position of the samples along the first two PLS axes. The percentages of variance explained by each axis are reported. (**B**) Variate loadings for the first two axes. Phe, phenylalanine; Asn, asparagine; Gaba, γ-aminobutyrate; Arg, arginine; Asn, asparagine; His, histidine; Val, valine; Pro, proline; Met, methionine; Gly, glycine; Ala, alanine.

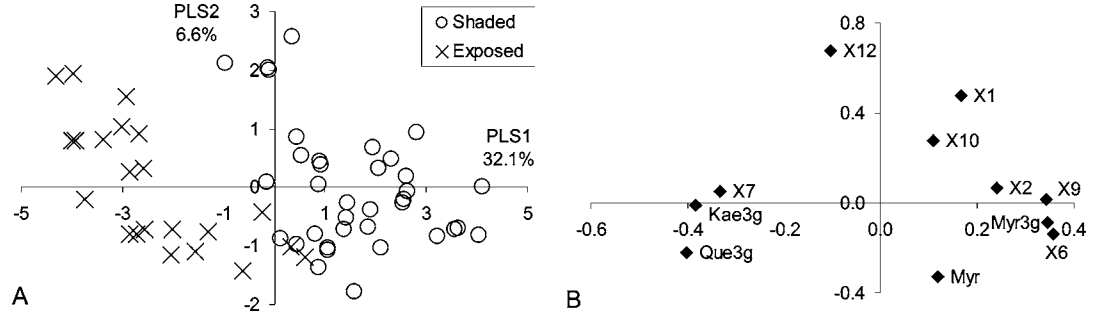

Figure 7. PLS discriminant analysis applied on phenolics (flavonols) of pulp extracts of 22 sun-exposed and 38 shaded berry samples sampled at the mature stage. (**A**) Position of the samples along the first two PLS axes. The percentages of variance explained by each axis are reported. (**B**) Variate loadings for the first two axes. X1, X2, X6, X7, and X10 are unknown flavonols; Myr3g, myricetin-3-glucoside; Myr, myricetin; Kae3g, kaempferol-3-glucoside; Que3g, quercetin-3-glucoside.

6A). The first score explained 20.8% of total variance, and the second score explained 15.1%. The main loadings of the first score (**Figure 6B**) were determined by arginine, phenylalanine, asparagine, and GABA on the positive side (shaded berries) and histidine, valine, proline, and methionine on the negative side (exposed berries). The second PLS score separated samples within the groups and was determined by arginine, alanine, and methionine on the positive side and glycine on the negative side.

PLS applied to amino acid data of skin extracts discriminated poorly the two groups of berries (data not shown). The first score explained 29% of total variance, the second one explained 17%. The first PLS score was loaded by alanine, GABA, leucine, and glutamate on the positive side. No major amino acid contributed to the negative side of the first score since only histidine, methionine, aspartate, and proline contributed weakly to the negative loading of the score 1.

Phenolics in Pulp and Skin Extracts of Grape Berries. PLS analysis was applied on the data of phenolics from the pulp extracts of shaded or exposed berries. The two groups were clearly separated by the first score (**Figure 7A**), explaining 32% of total variance. The variables which contributed to this score are shown on **Figure 7B**. Flavonols, kaempherol-3-glucoside, and quercetin-3-glucoside were the main variables on the negative side and myricetin-3-glucoside with some unidentified phenolics on the positive side.

PLS analysis applied on anthocyanins and flavonols data of skin extracts separated the exposed and shaded groups (**Figure 8A**). The first PLS score explained 31% of total variance

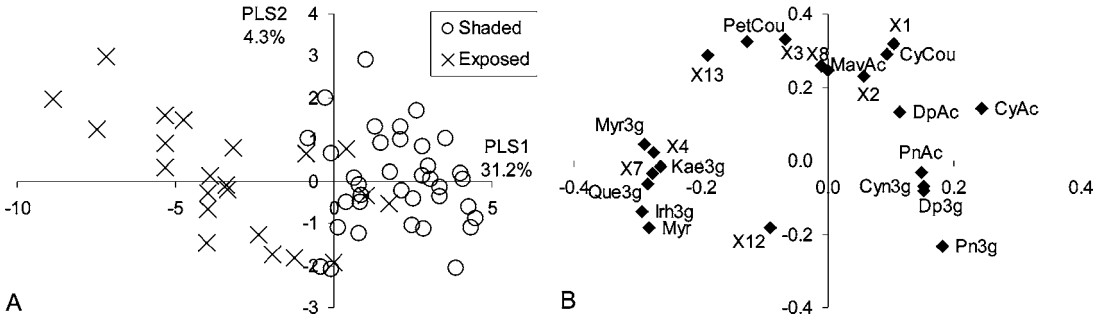

Figure 8. PLS discriminant analysis applied on phenolics (anthocyanins and flavonols) of skin extracts of 22 sun-exposed and 38 shaded berry samples sampled at the mature stage. (**A**) Position of the samples along the first two PLS axes. The percentages of variance explained by the axis are reported. (**B**) Variate loadings for the first two axes. X1, X2, X6, X7, and X10 are unknown flavonols; Myr3g, myricetin-3-glucoside; Myr, myricetin; Kae3g, kaempferol-3-glucoside; Que3g, quercetin-3-glucoside; Irh3g, isorhamnetin-3-glucoside; CyAc, cyanidin-3-glucoside acetate; Pn3G, peonidin-3-glucoside; Cyn3g, cyanidin-3-glucoside; Dp3g, delphinidin-3-glucoside; DpaAc, delphinidin-3-glucoside acetate; Cycou, cyanidin-3-glucoside coumarate.

Table 2. Metabolites and Minerals Contributing to Discrimination of Sun-Exposed and Shaded Berries Discrimination[a]

variable name	origin	definition
Mal	pulp	malate
K	pulp	potassium
DO360	skin	flavonols (optical density 360 nm)
N tot	pulp	total soluble nitrogen
S2.8	pulp	^{1}H NMR resonance (organic acids)
S4.4	pulp	^{1}H NMR resonance (organic acids)
4.1	pulp	^{1}H NMR resonance (fructose)
0.98	pulp	^{1}H NMR resonance (aliphatic compound)
1.26	skin	^{1}H NMR resonance (aliphatic compound)
5.42	skin	^{1}H NMR resonance (sucrose)
6.1	skin	^{1}H NMR resonance (aromatic)
2.38	skin	^{1}H NMR resonance (proline)
Phe	pulp	phenylalanine
Arg	pulp	arginine
His1	pulp	histidine
Val	pulp	valine
Ala	skin	alanine
Gaba	skin	GABA
His2	skin	histidine
Met	skin	methionine
X6	pulp	unidentified flavonols absorbing at 360 nm
Myr3g	pulp	myricetin-3-glucoside
Que3g1	pulp	quercetin-3-glucoside
Kae3g	pulp	kaempferol-3-glucoside
CyAc	skin	cyanidol-3-glucoside acetate
Pn3g	skin	peonidin-3-glucoside
Irh3g	skin	isorhamnetin-3-glucoside
Que3g2	skin	quercetin-3-glucoside

[a] List of the selected variables having the two highest loadings on both sides of the first two axes from the seven PLS discriminant analyses carried out on pulp and skin mineral and metabolite contents from shaded and sun-exposed berries (**Figures 3**–**9**).

(**Figure 8B**) with several flavonols contributing to the negative side and anthocyanins to the positive side. However, malvidin-3-glucoside, the most abundant anthocyanin in red vine varieties, did not significantly contribute to the loading of the first score.

Discriminant Analysis Based on the Most Discriminating Variables. To identify the most significant variables related to the light effect on grape berries, a composite data set was made up with the two most significant variables on the positive and the negative side of the first score for each previously shown discriminant analysis (**Table 2**) for pulp and skin together. This new data set allowed us to compare the relative capacity of these selected variables to separate the groups of shaded from that of sun-exposed berries. A PLS discriminant analysis was carried out on this new set of 28 variables and 60 samples. This selection was done to avoid an excess of variables compared to the number of samples and lessened the risk of PLS model overfitting (*23*). **Figure 9A** shows the data plot for the two first scores. The first score accounted for 31% of total variance and clearly separated the groups. The variables that contributed to the first score on the positive side were related to berry acidity: S2.8 and S4.4 are pulp NMR variables related to organic acid resonances, malate measured enzymatically on pulp extracts. On the negative side of the score, all variables related to flavonols were found associated with histidine and NMR variables (4.1 ppm corresponding to fructose + proline and 0.98 ppm corresponding to isoleucine + leucine + valine).

Sorting of a Test Data Set from Berries Sampled Independently. Twenty berry samples collected from sun (8 samples) and shaded (12 samples) locations in the canopy were used to test the discriminant analysis performed previously with the analytical data of the initial sampling. After each PLS, a linear discriminant analysis using the first two axes was calculated, performed, and saved. The 20 samples were tested with the eight models issued from the PLS analysis (**Table 3**), and the squared Mahalanobis distances of each data set of the test sample to the center of each group (sun and shaded) were calculated. PLS analysis carried out with maturation analytical data, NMR data, or HPLC amino acid data gave an erratic repartition of the test samples between the sun and shaded groups. HPLC phenolics data from skin made it possible to sort seven out of eight (87%) samples of berries from sun-exposed clusters after full leaf removal. Phenolic data from the pulp made it possible to sort 62% (five out of eight samples) of the sun-exposed clusters.

DISCUSSION

Response of Classical Maturity Analytical Data to Light Exposure. Exposed berries were smaller, contained less organic acids and nitrogen but more sugars. The skin extract showed a higher optical density in the near-UV (360 nm) attributed to flavonols. These analytical data are not independent. Smaller berries facilitate the concentration of sugars and amino acids. The diminution of organic acid is explained by the higher temperature of exposed berries (*8*). The increase of the optical density of the skin extract in the near-UV is explained by the photochemical effect of the light effect on flavonol content (*8*, *10*).

Discriminant Metabolites for Light Exposure. Partial least-squares analysis was based on sample grouping according to microclimatic measurements. Two groups were identified as shaded and sun-exposed berries. They were made up with berries sampled in the different positions in the vineyard (east or west side), outside, inside, or opposite to the clusters, not or partly

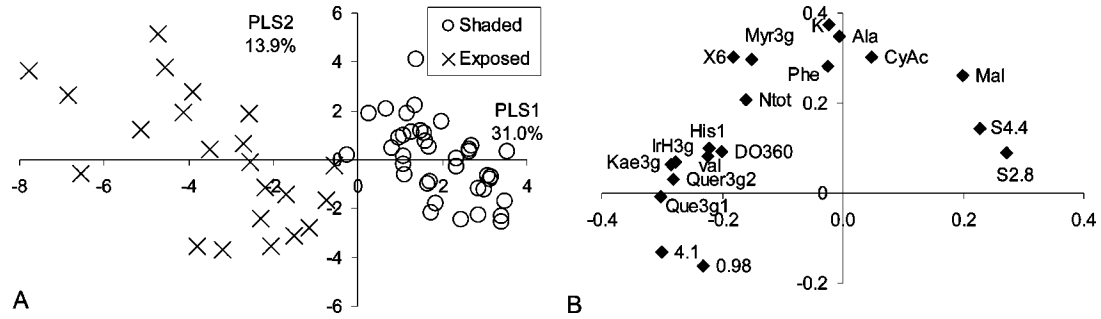

Figure 9. PLS discriminant analysis applied on selected variables of the four most discriminant metabolites or minerals from pulp and skin extracts of 22 sun-exposed and 38 shaded berry samples collected at the mature stage. (**A**) Position of the samples along the first two PLS axes. The percentages of variance explained by each axis are reported. (**B**) Most significant variate loadings for the first two axes are as follows. Variates from pulp metabolites: X6, unknown flavonol; Myr3g, myricetin-3-glucoside; Kae3g, kaempferol-3-glucoside; 0.98, 4.1, NMR buckets leucine, sugar, S4.4, and S2.8, respectively. NMR buckets from organic acids: Mal, malate; Phe, phenylalanine; His1, histidine; DO360, optical density of ethanol extract at 360 nm (flavonols); N tot, total soluble nitrogen; K, potassium. Variates from skin metabolites: Que3g, quercetin-3-glucoside; Irh3g, isorhamethyl-3-glucoside; Ala, alanine; CyAc, cyanidol-3-glucoside acetate (see **Table 3**).

Table 3. Linear Discriminant Analysis on Eight Data Sets from 20 Berries Sampled in Shaded or Exposed Position[a]

analytical data	classical analyses	NMR skin	NMR pulp	phenolics skin	phenolics pulp	amino acids skin	amino acids pulp	synthetic data set
exposed <ind;l>% correct	25	12.5	5	87.5	62.5	28	50	62.5
shaded <ind;l>% correct	92	83	83	100	82	54	25	100

[a] The percentage of correct classification of the berries by application of PLS discrimination models for the eight analytical data sets is reported. Berries are classified by calculating the Mahalanobis distance from the centers from the sun and shaded groups. The percentage of the berries correctly classified as exposed or shaded is reported after different PLS discriminant analysis. Skin phenolics are the most efficient analytical data set to separate exposed and shaded berries.

shaded by leaves. The sun-exposed and shaded batches were made, respectively, of five and eight different positions repeated five times. This sampling allowed us to collect data on the sun exposure effect on a population of berries which carries the natural variability observed inside a cluster, between clusters, and between plants in a vineyard.

A database was made up of seven analytical variable sets measured at maturity: classical mature berry analytical data (N, P, K^+, Ca^{2+}, Mg^{2+}, solid solubles, sugars, tartaric and malic acids, and total anthocyanins, total flavonols), 25 phenolics, 190 ^{1}H NMR spectral domains, and 20 amino acids in pulp and skin, all able to separate the sun-exposed and the shaded groups by partial least-squares analysis and linear discriminant analysis.

The most significant metabolites that contributed to the separation of the shade and sun berries were the flavonols (quercetin-3-glucoside, kaempferol-3-glucoside, myricetin-3-glucoside, and isorhamnetin-3-glucoside) in pulp and skin. This confirmed the already reported light effect on berry flavonols (*10*). Flavonols are synthesized in the outer part of the skin to screen UV radiations (*8*). Their accumulation would be induced by a light stress. But a temperature effect cannot be excluded because kaempferol-3-glucoside and quercetin-3-glucoside are also increased in the pulp of sun-exposed berries even though the UV light is fully filtered by the skin (*19*). In the pulp, sun exposure has mainly a temperature effect. Myricetin-3-glucoside showed an opposite behavior in skin and pulp. It is higher in the pulp and lower in the skin of shaded berries compared to sun-exposed ones. It can be suggested that the synthesis of flavonols is regulated by both light and temperature with a specific pattern for each compound. In a berry, the skin is more exposed to light than pulp when the temperature gradient is small, due to a high thermal conductivity of water in the fruits. Few other unknown compounds absorbing at 360 nm were significantly increased by light exposure. This pattern may reflect a complex response combining light stimulation of the synthesis and temperature-activated degradation of flavonols in sun berries.

Anthocyanin pattern is also clearly affected by light exposure. Malvidin-3-glucoside, the most abundant anthocyanin, was not affected by the lower light exposure level in this experiment (shaded position in the canopy). But shaded berries contained more peonidin-3-glucoside and cyanidin-3-glucoside acetate. These two anthocyanins follow a separate pathway from malvidin-3-glucoside related to the activity of the flavonoid 3′-hydroxylase and flavonoid 3′5′-hydroxylase (*40*). It has been shown that darkness significantly reduces the amount of anthocyanins (*41*), but clearly the amount of light in the shaded part of the canopy is enough for the synthesis of the malvidin-3-glucoside. High temperature induces a selective loss of grape anthocyanins by degradation (*8*). But the present data confirms the absence of the temperature effect on malvidin-3-glucoside amounts. Little is known about the in vivo catabolism of berry anthocyanins. Glucosidases which degrade anthocyanins are induced during the maturation of the berries, but there is no information on their selective affinity concerning the different anthocyanins (*42*). Light exposure of the berries reduced the amount of the intermediary anthocyanidins of the delphinidin branch (and their esterified forms) and cyanidin-3-glucoside and peonidine-3-glucoside in the cyanidin branch (*40*).

Malate and NMR spectral domains of organic acids content were very significant variates related to light exposure. A high temperature increases berry respiration which is partially fed by malate degradation through malic enzyme (*43*).

Amino acid content in pulp and skin showed different patterns in response to sun exposure. The pulp of exposed berries contained more histidine, valine, and proline. These same berries contained more total nitrogen. The pulp of shaded berries contained more arginine, phenylalanine, asparagine, and GABA.

Phenylalanine, precursor of the phenylpropanoid pathway, increased. This increase can be related to a lower biosynthetic activity of the flavonol pathway in shaded fruits. Arginine is the main storage amino acid in grape followed by proline (*44*). In exposed berries, less arginine and more proline were found. In exposed berries the arginine/proline balance was modified. A differential effect of temperature on the partition between proline and arginine from the ornithine pathway can be suggested (*44*). More nitrogen was stored in proline in exposed berries than in shaded ones. This observation is consistent with a significant biosynthesis of proline from the ornithine pathway as proline accumulation is not controlled by the Δ^1-pyrroline-5-carboxylate synthetase activity (*45*). Histidine and valine were very significant markers of berry microclimate. They are increased by light exposure, but little is known about their role and metabolism in grapes. Valine change can be related to the report of Broeckling et al. (*46*), who showed that valine and other branched amino acids were accumulated after methyl jasmonate treatment in *Medicago truncatula* and a change in the phenylpropanoid pathway by the same elicitor in the berry skin (*47*). Histidine accumulation in *Arabidopsis* has been explained by a modification of the feedback control of the pathway at the ATP phosphoribosyl transferase step (*48*). Alternatively, histidine metabolism could be involved in the control of cytosolic pH (*49*). The amino acid patterns in skin and pulp were different. In skin and pulp GABA was more abundant in shaded berries. GABA and alanine are associated with abiotic stress and cytosolic pH control (*50*). But GABA is also related to the C/N balance and defense against insects (*51*). The GABA response is not explained by a temperature stress which is lower on shaded berries. Its contribution to tissue-specific N management in skin is a more attractive hypothesis. A berry pulp cope with the nitrogen import coupled with transpiration (xylem sap) and sugar carried by the phloem sap GABA can store a significant amount of N in skin when pulp can also store the excess of imported N into arginine (*52*). However, GABA is more abundant in shaded berries that contain less total soluble nitrogen. A change in the free amino acid content was related to an altered feedback control of the biosynthetic pathway (*53*). A detailed study of the glutamate decarboxylase activity, cytosolic pH control, and/or the protein biosynthesis and degradation activity will bring new information on the role of GABA in grape berries in relation to temperature (*50*).

Discriminant Capacity of Nontargeted versus Targeted Metabolic Profiles? ^{1}H NMR profiles were used as nontargeted analytical data. The profiles were described by 190 buckets representative of the proton amounts in the diversity of their molecular environment. The discriminant analyses were performed on these basic quantitative data. The use of untargeted data offered the possibility to identify nonexpected compounds. The contribution of sucrose to the discrimination is an original information. This sugar is not usually quantified in mature grape berries. In skin extracts only two unattributed resonances localized in the aliphatic zone are related to shade. All the other significant resonances from the NMR spectra were found in the organic acid and amino acids spectral domains. Some of them were confirmed by HPLC analysis. The untargeted approach did not improve the efficiency of the discrimination between the groups. This is probably due to the relative low sensitivity of the ^{1}H NMR technique and the overlapping of the signals from different metabolites (*26*).

Metabolic Profiling of Sun-Exposed and Shaded Berries. Discriminant analysis based on the total ^{1}H NMR signature made it possible to separate the two berry groups although 10% or less of total variance was explained by the first score for pulp and skin, respectively. The NMR buckets are representative of a wide range of metabolites, amino acids, organic acids, sugars, and aromatic compounds. However, this capacity did not improve the separation of the different groups compared to that of other analytical data. Since the stability of the position of some signals can be questioned, mainly for organic acids (*54*), summing the signals of the organic acid regions was used. The very high amount of soluble sugars, their extremely high number of overlapping signals, and the lack of sensitivity in the aromatic region considerably reduced the efficiency of the method (*26*).

Despite these limits the ^{1}H NMR data showed a significant contribution of proline and valine confirmed by targeted amino acid analysis. The resonance attributed to protons from organic acids was clearly related to shaded berries. NMR results are consistent with targeted analytical data and showed that sucrose in skin was more abundant in shaded berries. These results have not been observed previously. Sucrose can be an interesting indicator of berry status.

A synthetic data set was made up with the four most significant loadings from each analytical set. It will be used to identify the most discriminant metabolic analytical variables for sun-exposed and shaded grape berries. The PLS discriminant analysis based on these 28 selected variables showed that most of the variance induced by grape exposure was explained by an accumulation of flavonols (quercetin 3-glucoside, kaempferol-3-gl, isorhamnetyl-3-gl) in pulp and/or skin. Valine and histidine in the pulp were also significant markers of light exposure. Organic acid, mainly malate content, was also a significant indicator of exposed berries. But it must not be used alone because organic acids are related to berry temperature which is not specifically related to light exposure.

Data sets from berries collected in the same field experiment but not used for the PLS and linear discriminant analysis had been used to check the capacity of the model to sort out sun-exposed from shaded berries (**Table 3**). Skin and pulp phenolic profilings gave the best results. The other analytical data set did not allow us to sort berries efficiently. The metabolic profile which associates phenolics (HPLC), sugars (NMR), amino acids (HPLC), and organic acids (NMR) gave a poor result compared to that of phenolics alone.

In conclusion, the most significant effect of light exposure observed in this experiment on grape is on flavonol content. The metabolic flux in the flavonoid pathway is known to be regulated by red and ultraviolet light-activated photoreceptors in several plant species (*55*). These compounds are good indicators of light exposure because they are closely controlled by photochemical reactions and are probably secondarily affected by the increase of temperature related to light exposure. Anthocyanins which are photochemically regulated are also affected by a temperature increase (*20*). Their response is more ambiguous. And finally, organic acids which are mainly affected by temperature (*9*) were not very discriminating for light exposure of the clusters. Although it was not possible to separate the light effect from the temperature effects in this field experiment, the comparison of sun-exposed or shaded metabolic profiles of pulp and skin allowed us to separate apparent light from temperature effects on metabolites. Flavonols were more representative of a light effect except myricetin 3-glucoside. Organic acids, valine, histidine, proline, and arginin in the pulp were more affected by temperature. The major anthocyanins are both increased by light but inhibited by temperature. They

were not significant markers. Conversely, secondary anthocyanins and a few acylated forms were favored by low light and contributed to the metabolite profiling discrimination of grape berries. In practice, these results could be applied to define leaf removal strategy in the vineyards in function of latitude, climate, row orientation, and grapevine architecture. The objective would be to balance light and temperature effects on the berry metabolic profile in the natural environment of the vineyard. A discriminating tool based on PLS discriminant analysis and a database is now available to characterize berries exposed to unknown light environment during maturation. It will be improved by adding data from other vintages to take in account the interaction between the climate in the vineyard and the microclimate in the canopy. It points out metabolites markers of sun exposure but raises many questions about the physiological origin of these metabolic patterns.

ACKNOWLEDGMENT

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