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Microclimatic influences on grape quality

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Ort, Datum Matthias Friedel

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[1]Friedel M., Patz C.D. & Dietrich H. 2013. Comparison of different measurement techniques and variable selection methods for FT-MIR in wine analysis. *Food Chemistry* **141** (4), 4000-4007

[2]Friedel M., Stoll M., Patz C.D., Will F. & Dietrich H. 2015. Impact of light exposure on fruit composition of white 'Riesling' grape berries (*Vitis vinifera* L.). *Vitis* **54** (3), 107-116

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Chapter 1

General Introduction

The Grapevine Canopy Microclimate

The fact that microclimatic conditions play a crucial role in the production of quality agricultural produce, including wine grapes, has been known to agricultural experts since the classical age (Columella). While the definitions of the geographic extent of microclimatic phenomena vary to a certain degree to this day (Foken, 2016), there is general consent that all climatic variations within stands of crops belong to the micro-scale of climatic phenomena. Microclimatic phenomena usually build up in ground-near air layers and are mainly influenced by the present surfaces, including relief and vegetation cover, and their physical properties (Orlanski, 1975). Thus, great differences of temperature and wind speed can occur in plant populations as compared to ambient temperature. In viticulture, the term microclimate has mostly been used to describe the microclimate within grapevine canopies (Smart et al., 1985a). This definition takes in account temperature, relative humidity, wind speed and, as one of the most important parameters, the penetration of radiation into the canopy, the latter of which is not included in most definitions of climatic phenomena.

The canopy microclimate is influenced by a variety of factors. These include trellis system, row spacing, canopy height, canopy width, row orientation, shoot density, vine vigor, rootstock and cultivar, canopy distance to ground, soil type and undergrowth (Hoppmann, 2010; Smart, 1973; Smart et al., 1985a). Most of these factors, like vineyard architecture and plant material, can only be influenced by long-term cultural decisions. With increasing awareness of the microclimatic effects on grape quality and plant health, strategies to manipulate the microclimate of grapevine canopies have been developed by growers and researchers. These cultural practices have gained worldwide attention and have to be considered as main contributors to canopy microclimate. Microclimate manipulation practices include the removal of leaves in or above the bunch zone, shoot removal and trimming and hedging of the canopy (Müller et al., 1999; Smart and Robinson, 1991). As almost all quality traits of winegrapes have been reported to respond to

one or more climatic factors, experiments about their impact have been published as early as 1846 (Kern and v. Babo, 1846).

Significance and Evolution of Perception

Intense research on grapevine canopy microclimate, however, was only conducted in the 20th century, when the breeding of vigorous rootstocks, the rise of mineral fertilization and the establishment of vineyards on fertile soils led to increasingly dense canopies. Due to their unfavorable microclimatic conditions, dense canopies typically led to problems like an increased disease pressure, a lower grape and wine quality and an excessive amount of shaded leaves. Awareness of this problem rose in the 1960s, when professor Nelson Shaulis of Cornell University began to promote a better light interception of the grape bunches as a tool to overcome these problems. He and his disciples, among them Alain Carbonneau from France, Richard Smart from Australia and Giovanni Cargnello from Italy, developed a variety of vine training systems with the aim of improving the penetration of sunlight into the canopy and the sun exposure of the grape bunches. These training systems include Shaulis' Geneva double curtain, the Smart-Dyson system and Carbonneau's Lyre (Carbonneau, 2009).

Although this first generation of microclimate researchers kept promoting novel trellis systems to optimize canopy microclimate and fruit quality, none of these novel systems gained sufficient traction in the wine industry compete with the popular vertical shoot positioning (VSP) system, which increasingly replaced traditional vine training systems. The labor-intensive, hardly mechanisable management of many of the novel trellis systems limited their application to ultra-premium wine production. When the emergence of a novel phylloxera biotype led to the replanting of large vineyard surfaces in California in the mid 1980s, many of the vineyards trained to a traditional "California sprawl" trellis system were replaced by VSP trained vineyards (Dokoozlian, 2009). This development was accelerated by the producers of agricultural machinery, who oriented their developments towards VSP-type systems. Although these novel trellising systems did not prevail in the wine industry, the work of pioneers like Kliewer, Smart and Buttrose, carried out mostly in anglophone countries, are still considered the fundamentals of modern canopy management. Their work spanned the factors influencing canopy microclimate (reviewed by Smart et al., 1985) and berry temperature (Smart and Sinclair, 1976), observations of light and temperature effects on grape quality compounds

such as anthocyanins (Buttrose et al., 1971; Kliewer, 1970a), amino acids (Kliewer and Ough, 1970) and organic acids (Lakso and Kliewer, 1975) and the first applications of targeted canopy manipulation (Kliewer, 1970b; May et al., 1969).

Knowledge about the benefits of canopy management continued to increase during the 1970s and 1980s, and in 1987 the first conference focusing purely on the targeted improvement of the canopy microclimate by canopy manipulation and vigor management was held at UC Davis, California. In practice, most canopy management strategies remained restricted to the production of high quality red winegrapes, as the high labor input for accurate canopy management, which was then generally done by hand (Gubler et al., 1987), prevented the further advance into the production of lower quality red or white grapes. In quality red grape and table grape production, where fungal diseases lead to severe economic losses, the phytosanitary benefits of canopy management and its action against *Botrytis cinerea* infection were a main driver of its application (Gubler et al., 1991; Koblet, 1987). The labor input requirement further confined leaf removal to periods with little other vineyard work, which was usually during the ripening phase. This - and the cost and complexity of grape and wine aroma analysis - might explain the amount of work dedicated to the research of microclimatic effects around veraison on basic red grape quality, while there were little studies in this regard conducted on white grapes. The scope of canopy manipulation started to change when the scientific and practical interest in leaf removal encouraged technical progress towards the development of mechanized leaf removal.

Mechanized leaf removal

Attempts to mechanize leaf removal were made from the 1960s onwards. However, solutions like chemical or thermal leaf removal were not accepted by the wine industry. The breakthrough for the widespread application of microclimate manipulation techniques was laid in the late 1980s, when the first machines to conduct targeted leaf removal by suction and cutting became available (Gubler et al., 1991). These machines reduced the labor demand of leaf removal from 40-60 working hours ha^{-1} to only 3-6 working hours ha^{-1}. In parallel, the cost of leaf removal was lowered by 30-80 % depending on the fixed cost degression. Mechanization increased the clout to conduct leaf removal in vineyards and hence enabled the application of this technique to entire farms. Up to the present day, three larger operating

principles of leaf removal machines evolved: leaf pullers which use a suction fan in combination with two counter-rotating rollers, leaf cutters which use a suction fan in combination with rotating blades, and devices which use air pulses to crush leaves. The different properties of the respective leaf removal machines are displayed in table 1. The comprehensive introduction of mechanized leaf removal led to decoupling of the leaf removal process from the period of low labor intensity during berry maturation. Nowadays, three typical leaf removal strategies are established: An early leaf removal, which is conducted before bloom with the aim of decreasing the number of berries per bunch by limiting pre-bloom assimilate supply and thus induce a lower rate of flowering; Leaf removal shortly after bloom with the aim of reducing berry size by limiting assimilate supply after bloom; And the traditional leaf removal at around veraison with the aim of improving microclimate. The general association of leaf removal with beneficial effects, combined with the low costs of mechanized leaf removal also encouraged the excessive use of leaf removal in unsuitable vineyards or situations, which often resulted in poor grape quality. This led to the critical review of the work formerly conducted on canopy management. One of the critical remarks about the early canopy management work was that sunlight onto grape bunches was considered beneficial for nearly all parameters at any stage of grape growth in any viticultural region worldwide, often with no regard to the grape cultivar.

Table 1. Operating principles, technical data and costs of modern leaf removal techniques, adapted after Müller and Walg (2013)

Operating principle	Suction/Cutting	Suction/Plucking	Blowing /Plucking	Tearing by air pulse	Manual leaf removal
Company	Pellenc, Ero, Clemens	Binger Seilzug, KMS Rinklin	Freilauber	Siegwald, Collard	-
Technical data					
Operating speed (km/h)	4-7	4-6	3,5-5	1,5-3	-
man-hours/ha (one side)	1,5	1,5-2	2-2,5	3-4	17-40
man-hours/ha (both sides)	3	3-4	4-5	6-8	30-70
Costs					
Aquisition (€)	6000	8000-9000	8000	ca. 20.000-25.000	-
€/ha (10 ha, one side)	142	190	210	428	300-500
€/ha (20 ha, both sides)	104	138	158	296	

This approach was reflected in Richard Smarts statement that his vineyard scorecard, recommending that 60 % or more of all bunches be exposed, “is now being used as a routine vineyard quality assessment guide by leading wineries in New Zealand, Australia, and California” (Smart and Sharp, 1989). Clearly, such generalizations did not take into account the enormously different responses to leaf removal or other microclimate

manipulations among the macroclimates found in different growing regions as well as the differences among cultivars or developmental stages. To overcome these gaps of knowledge, research about canopy management broadened its aims and scope during the 1990s. Research then included the timing during which canopy manipulations were applied (Dokoozlian and Kliewer, 1996) and the effect of different light intensities and temperatures (Spayd et al., 2002), often on multiple cultivars (Bergqvist et al., 2001). Much of the effort in microclimatic research during this period, however remained limited to the field of phenolic compound synthesis in red varieties. In the new millennium, molecular biology and modern analytical tools allow to gain an in-depth understanding of the effects of microclimate manipulation on grape quality and vine physiology.

Microclimatic effects on grape quality

The manipulation of the canopy microclimate has originally been performed to improve plant health. Grapes and leaves in less dense canopies dry faster after morning dew or rain events due to a better airflow through the canopy and direct radiation (Smart, 1985). An improved microclimate also delays the spread of fungal diseases by the direct fungicidal effects of sunlight (Gnanamanickam, 2002). Only recently it has been discovered that sunlight influence also stimulates salicylic acid and jasmonate mediated plant systemic acquired resistance and induced systemic resistance to pathogens and herbivores (Agrawal et al., 2012; Griebel and Zeier, 2008; Vernooij et al., 1994). Plant defense against pathogens is associated with the accumulation of secondary metabolites (Gnanamanickam, 2002). Many of these secondary metabolites are considered valuable in terms of product quality and display important health benefits in human nutrition (Teixeira et al., 2014). It has long been recognized that the microclimate interacts strongly with the secondary metabolism of grapevines. In addition, the primary metabolism of grapevines is also influenced by microclimatic conditions. Light and temperature effects on the respective classes of metabolites will be outlined in the following section.

A recent genomic study has shown that about 18 % of the transcripts modulated during grape berry development are sensitive to environmental factors (Dal Santo et al., 2013), rendering the microclimate as one of the most important drivers of grape phenotypic plasticity. Light generally plays an important role in fruit ripening. The influence of irradiation on the most crucial parameter defining fruit ripening, the accumulation of sugars,

strongly varies among different cultures. In grapes, the contribution of fruit photosynthesis to total soluble solid (TSS) concentration is negligible (Pandey and Farmahan, 1977). In fact, grape chlorophyll concentration decreases strongly during ripening. However, it has been hypothesized that light plays a role in determining the carbohydrate sink strength of grapes (Dokoozlian and Kliewer, 1996). Investigations about light influence on grape ripening have employed artificial and natural methodologies of microclimate manipulation, and have led to sometimes contradictory results. Dokoozlian and Kliewer (1996) found that artificially shaded fruit had a lower concentration of TSS, in accordance with results of Reynolds and Wardle (1989a) obtained under natural canopy shade. In contrast, Reynolds and Wardle (1989b) found higher TSS concentrations in dense canopies with low fruit exposure as compared to more open canopies with higher fruit exposure obtained by heading and leaf removal. Similarly, Skinkis et al. (2010) found no differences in TSS concentration between exposed and artificially shaded grapes. The temperature regime under which bunches ripen, however, appears to play a role in sugar accumulation. Using an air-blower to heat and cool individual bunches, Cohen et al. (2012) have shown that a compression of the diurnal temperature range of bunches accelerates grape ripening.

The microclimatic influence on the concentration of organic acids differs strongly between the respective compounds. The decrease of tartaric acid concentration during maturation originates from a dilution effect of the growing berry mass (DeBolt et al., 2008; Dokoozlian and Kliewer, 1996), and neither berry temperature nor direct radiation influence its degradation. In contrast, a clear temperature effect has been shown for the degradation of malic acid. Malic acid is respired during maturation, and the optimum temperatures of the malic enzyme catalyzing its respiration show optimum temperatures of above 40 °C (Lakso and Kliewer, 1975).

Temperature and radiation effects on pH-values and total titratable acidity (TA) are less clear. It has been shown that leaf removal in hot climates helps winemakers to maintain a lower juice pH (Martinez de Toda and Balda, 2014), which may be explained by a lower potassium concentration found in juice originating from fruit shaded by the canopy (Smart et al., 1985b).

Similar to organic acids, the effect of microclimatic conditions on the concentration of different phenolic compounds can’t be generalized. For example, it appears that light or temperature do not influence the concentration of grape hydroxycinammic acids (Šuklje et al., 2012). On the

other hand, the biosynthesis of flavanols seems to be influenced by irradiation during and shortly after flowering (Koyama et al., 2014).

Flavonol synthesis is specifically and rapidly induced by UV light (Kolb et al., 2003) via the transcription factors VvMYBF1 (Czemmel et al., 2009) and the expression of flavonol synthase (FLS) genes with their synthesis peaking at flowering and ripening (Downey et al., 2004). It has been pointed out that gene expression of flavonol biosynthetic genes is coordinately controlled. UV radiation (Adrian et al., 2000) also has a strong influence on stilbene synthesis. Stilbene synthesis in grapes is controlled by the transcription factors VvMYB14 and VvMYB15 (Höll et al., 2013). Gene expression of these transcription factors and, correspondingly, the stilbene synthases VvSTS25/27/29 and VvSTS41/45 have been shown to be up-regulated by light (Höll, 2014). Microclimatic conditions also influence the concentration of anthocyanins in grapes (Dokoozlian and Kliewer, 1996), with sunlight up-regulating their synthesis (Matus et al., 2009) and elevated temperatures accelerating their degradation (Mori et al., 2007). It has also been shown that the a compression of the diurnal temperature range changes the profiles of anthocyanins and flavanols during berry ripening (Cohen et al., 2012).

Although it appears that amino acid concentrations in grape berries are higher in hot climates than under cooler conditions, solar radiation appears to have a negative influence on amino acid and, more generally, nitrogen compound accumulation in grapes. This effect seems to be related to radiation rather than to berry temperature, as a study utilizing UV-filtering materials has shown (Schultz et al., 1998). Another study found smaller effects of UV-B radiation, but large effects when bunches were sheltered by an intact canopy without leaf removal (Gregan et al., 2012).

The concentration of some important volatile compounds such as norisoprenoids (Marais et al., 1992) has been shown to decrease by shading, while others, like C6 compounds or non-terpenic alcohols, do not seem to be affected (Bureau et al., 2000b). Other aromatic compounds, such as the methoxypyrazines, which confer a distinct, often undesired taste of grass or green capsicum to wine, are subject to photodegradation (Hashizume and Samuta, 1999). The picture for terpenoid synthesis in grapes is less clear, as data have been published which show lower terpenoid concentration in sun-exposed as compared to (naturally or artificially) shaded grapes (Bureau et al., 2000b; Scafidi et al., 2013), while other studies indicate the opposite effect (Reynolds and Wardle, 1989a; Skinkis et al., 2010). On a molecular

basis, Gil et al. (2012) have shown an UV-B induced increase of terpene synthase enzyme activity in grapevine leaves.

While this short overview highlights the importance of microclimatic factors for grape health and quality, it also shows that there are still contradictions and a lack of knowledge about the microclimatic influence on a number of compounds within different varieties. This regards especially the impact of timing and intensity of microclimate manipulation on the quality of white grape cultivars.

Objectives

This Ph.D. project was conducted to combine new and efficient analysis tools with microclimate manipulation experiments to gain a deeper understanding of the metabolic processes influenced by microclimate manipulation on the white *Vitis vinifera* cultivar Riesling, which is the cultivar with the highest economic impact in Germany. Within these projects, co-variance data of berry optico-physical properties and grape quality traits should be investigated in order to evaluate the potential use of optical measurements in industrial applications. The Ph.D. project was divided into four parts to separate the respective aims:

Fourier-transform infrared spectroscopy (FTIR) combined with partial least squares (PLS) data analysis has been introduced to wine analysis at the beginning of the century. Provided FTIR calibrations are stable and accurate, FTIR represents a high-throughput analysis suitable for the rapid, cost-effective and environmentally friendly measurement of up to a dozen wine parameters simultaneously. While several different FTIR measurement techniques and variable selection procedures are used in the wine industry, these different approaches have as yet not been compared on an industry-specific matrix. As microclimatic experimentation typically requires the analysis of a large number of samples, the aim of the first part of this Ph.D. project was to optimize the FTIR calibration methodology by comparing different methods of variable selection on spectra obtained on several instruments employing different measurement techniques. It is displayed in Chapter 2.

The second part of the project, shown in Chapter 3, aimed at getting a better understanding of the effects of different irradiation regimes on grape composition of Riesling in the course of berry development. In this part of the project, the effects of different light regimes on the concentration of sugars, organic acids, berry skin phenolics and amino acids was investigated in the growing seasons 2011 and 2012. To achieve this, bunches shaded in opaque boxes and fully irradiated bunches were compared to bunches grown in a normal canopy without leaf removal. In the second experimental year, the application of these treatments was conducted at two different key points of berry development, fruit set and veraison, to increase knowledge about the effects of early defoliation, which is increasingly applied in European viticulture.

Another crucial aspect of grape quality is the aromatic composition of a grape, in which the terpenoid fraction plays a key role. Controversial results have been published about the role of the canopy microclimate for terpenoid accumulation in the grapes. Recent progress in molecular biological research revealed a large number of genes encoding for enzymes involved in the synthesis of terpenoids in grapevine, among them the first functional terpenoid glycosyl transferase. In the third part of the project, light effects on the expression of a number of these genes were investigated for the first time and compared to the expression of the flavonol metabolism, which is known for its light sensitivity. In parallel, the accumulation of the respective terpenoid and flavonoid metabolites was monitored. As controversial data have been published on the effects of light on terpenoid synthesis, the third part of the thesis aimed to clarify the role of light in terpenoid metabolism in grapevine. This part is summarized in Chapter 4.

Berry sorting applications have become increasingly popular in the wine industry. A new generation of berry sorting equipment has recently been introduced to the market. These berry sorting machines can discriminate berry fruit by their size, shape and reflectance in various parts of the electromagnetic spectrum. Berry color and berry size data acquired, but not published during the previous studies yielded evidence that berry color and berry size correlate with some grape quality parameters. These correlations were not linear and apparently no direct correlations, but rather co-variances caused by microclimatic effects. When applied to berry sorting, berry color and size appear to be promising parameters to separate high-quality from low-quality fruit. The final part of the study, introduced in Chapter 5, aimed at testing the hypothesis that berry sorting by size and color does indeed lead to differences in final wine quality. To test this hypothesis, a microfermentation trial of berries sorted by size and color was conducted. To exclude the sugar concentration as a biasing factor, the trial was conducted on berries with equal sugar concentration.

Chapter 2

Comparison of different measurement techniques and variable selection methods for FT-MIR in wine analysis[1]

[1]Friedel M., Patz C.D., & Dietrich H. 2013. Comparison of different measurement techniques and variable selection methods for FT-MIR in wine analysis. *Food Chemistry* **141** (4), 4000-4007

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Analytical Methods

Comparison of different measurement techniques and variable selection methods for FT-MIR in wine analysis

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ABSTRACT

For more than a decade, Fourier-transform infrared (FTIR) spectroscopy combined with partial least squares (PLS) regression has been used as a fast and reliable method for simultaneous estimation of multiple parameters in wine. In this study, different FTIR instruments (single bounce attenuated total reflection, transmission with variable and defined pathlength) and different variable selection techniques (full spectrum PLS, genetic algorithm PLS, interval PLS, principal variable PLS) were compared on an identical sample set of international wines and ten wine parameters. Results suggest that the single bounce attenuated total reflection technique is well suited for the analysis of ethanol, relative density and sugars, but less accurate in the analysis of organic acid content. The transmission instrument with variable pathlength shows good validation results for the analysis of organic acids, but less accurate results for the analysis of ethanol and relative density as compared to the other instruments. The transmission instrument with defined pathlength was well suited for the analysis for all parameters investigated in this study. Variable selection improved model robustness and calibration results, with genetic algorithm PLS being the most effective technique.

1. Introduction

In the food industry, compliance with new food and legal standards, as well as the demand for high-quality-products, require a close monitoring of the product over the whole production process (Christaki & Tzia, 2002). Therefore there is a need for a fast and reliable method for the quality control of the products. In the wine and beverage industry, spectroscopic methods in the NIR (Baumgarten, 1987; Garcia-Jares & Medina, 1997; Gishen & Dambergs, 1998) and MIR (Patz, Blieke, Ristow, & Dietrich, 2004; Patz, David, Thente, Kürbel, & Dietrich, 1999; Schindler, Vonach, Lendl, & Kellner, 1998; Soriano, Perez-Juan, Vicario, Gonzalez, & Perez-Coello, 2007; Tarantilis, Troianou, Pappas, Kotseridis, & Polissiou, 2008) ranges of the electromagnetic spectrum presented a part of the solution to these problems. IR-spectroscopic methods offer environmentally friendly, rapid and simultaneous analysis of a large variety of parameters without the need for costly and time-consuming sample preparation (Kessler, 2007; Lachenmeier, 2007). Absorption in the NIR region consists of weak overtones and combination bands mainly of C–H bonds. In the MIR region, fundamental stretching and bending vibrations of C–H, C–O, O–H and N–H bonds result in strong absorption (Nieuwoudt, Bauer, & Kossmann, 2008). The strong absorption in the MIR region is on one hand of analytical advantage, causing various information-rich sharp peaks in the MIR-spectrum. On the other hand, samples containing high amounts of water and organic compounds, e.g. wine or juice, are highly absorptive in the MIR region. Therefore it is not possible to use long light pathlengths in FT-MIR wine analysis. Most of the spectroscopic hardware for the analysis of liquids, however, is still based on measurements employing transmission measurements using CaF_2 cuvettes. The need for pathlengths in the order of a few micrometers leads to constructory and analytical problems, especially when dealing with highly viscous, abrasive or cloudy samples. A potential alternative to transmission measurements is the use of attenuated total reflection (ATR) FTIR spectroscopy. This technique, which was established in the 1960s by Harrick (1963) and Fahrenfort (1961), is based on the principle of evanescence, and is usually used for the analysis of highly absorptive liquid samples or surfaces (Gottwald & Wachter, 1997). In ATR spectroscopy penetration depth into the medium is dependent on the wavelength and the refractive index of the sample (up to 3 μm, dependent on the setup), but is much lower than

Abbreviations: FTIR: fourier transform infrared spectroscopy; MIR: mid infrared; NIR: near infrared; PLS: partial least squares; RMSEP: root mean square error of prediction; SEP, standard error of prediction; RPD: ratio of prediction to standard deviation; SB-ATR: single bounce attenuated total reflexion; LV: latent variable.

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in transmission-type-spectrometers (10–50 μm). Therefore, even the regions which show total absorption by water in transmission-type spectra are accessible for analysis in ATR-spectra.

Extraction of relevant information from the spectra can be achieved by linear multivariate calibration techniques such as PLS regression, multiple linear regression (MLR) or nonlinear methods such as artificial neural networks (ANN) or support vector machines (SVM) (Balabin & Smirnov, 2011). PLS is the most common calibration tool in spectroscopy (Wold, Sjostrom, & Eriksson, 2001). While in some cases the whole MIR-spectrum is used for calibration (FullPLS), a selection of relevant spectral areas (filters, features, variable subsets) is often performed before building a model. The aim of the variable selection is the identification of spectral regions which are important for the prediction of the analyte, while excluding noisy variables or such carrying little information about the analyte or important interferences. Nadler and Coifman (2005) show mathematical evidence that RMSEP in full-spectrum PLS-calibration is a function of spectral dimension (number of spectral variables) in a simulated data set, while the application of a variable selection algorithm prior to PLS produced calibrations with a stable RMSEP independent of spectral dimension. The algorithm used for the variable selection is of great importance for the result of the calibration (Hoskuldsson, 2001; Nadler & Coifman, 2005).

In this study the performance of two FTIR instruments employing transmission cuvettes with fixed and variable pathlength was compared to the performance of an FTIR-ATR instrument. Basis for the comparison were ten relevant wine parameters. For the comparison identical samples for calibration and model validation were used on all instruments. To check if different variable selection techniques are better suited for specific instruments and to put the instrument comparison on a basis of not just one model for each parameter, four different variable selection techniques were applied to the spectra, and a PLS model was built for every variable selection method.

2. Material and methods

2.1. Data set

The data set used for this study consisted of 166 international wines originating from different countries and included red, white and rosé and sparkling wines from the vintages 2008, 2009 and 2010 (Table 1). The data set was split into a calibration (2/3 or 108 wines) and a validation (1/3 or 58 wines) data set. Calibration and validation data sets are characterized in Table 2.

The parameters citric acid and acetic acid were not used in the comparison of the instruments and algorithms, as mean and standard deviation were low in our data set and unsatisfying calibration results for these parameters have been reported in literature (Cocciardi, Ismail, & Sedman, 2005; Cozzolino, Cynkar, Shah, & Smith, 2011; Patz et al., 1999, 2004).

Table 1
Proveniences and wine types of the samples used in this study.

Country	Red	Rosé	Sparkling	White	Total
Argentina	1				**1**
Australia	3			2	**5**
Austria	1			1	**2**
Brazil	3			1	**4**
Chile	5			2	**7**
France	11	3	1	1	**16**
Germany	6	1		9	**16**
Hungary				3	**3**
Italy	55	4	5	18	**82**
South Africa	9			1	**10**
Spain	3			3	**6**
USA	10			4	**14**
Total	**107**	**8**	**6**	**45**	**166**

2.2. Wine reference analysis

Reference analysis for the calibration models was conducted according to the international organization of vine and wine (OIV) compendium of international methods for grape and wine analysis (OIV, 2013). Together with the description of the reference methods, their repeatability (r) and reproducibility (R) values, derived from the same source, are denoted in brackets. Where no data were available for r and R in the OIV compendium, data were derived from the official method compendium for the analysis of foodstuffs after § 64 LFGB of the german law for foodstuffs, articles of daily need, and animal feed (*Amtliche Sammlung von Untersuchungsverfahren nach § 64 LFGB*, Analyseverfahren für die Untersuchung von Lebensmitteln, 2011). For glycerol, whereno data are available, the values were calculated with the Horwitz equation (Horwitz, 1982; Horwitz & Albert, 1996). Repeatability was calculated as 66% of the reproducibility value (Albert & Horwitz, 1997). For heteroscedastic precision values, the values given are calculated for the entire data set, including calibration and validation sets.

Relative density (r = 0.0001; R = 0.00013) was measured using an oscillation-type density meter (DMA 5000, Anton Paar GmbH, Graz, Austria).

Ethanol (r = 0.53 g L^{-1}; R = 1.39 g L^{-1}) determination was conducted by distillation of 50 mL of wine sample. The ethanol content was calculated from the density of the distillate, measured by an oscillation-type density meter (DMA 5000, Anton Paar GmbH, Graz, Austria).

Glucose (r = 0.15 g L^{-1}; R = 0.33 g L^{-1}), fructose (r = 0.18 g L^{-1}; R = 0.36 g L^{-1}), L-malic acid (r = 0.05 g L^{-1}; R = 0.09 g L^{-1}), acetic acid, citric acid and glycerol (r = 0.21 g L^{-1}; R = 0.32 g L^{-1}) were determined enzymatically (R-Biopharm) with a sequential analyser (Konelab 20 XTi, Thermo Scientific). Fermentable sugar (r = 0.33 g L^{-1}; R = 0.57 g L^{-1}) was calculated as the sum of glucose and fructose. Lactic acid (r = 0.11 g L^{-1}; R = 0.21 g L^{-1}) was calculated as the sum of L-(-)-lacic acid and D-(+)-lactic acid, which were determined enzymatically. The pH (r = 0.0317; R = 0.0476) was measured potentiometrically at 20 °C with a glass/calomel electrode.

Total acidity (r = 0.07 g L^{-1}; R = 0.30 g L^{-1}) was determined by titration to pH 7.0 with 0,33 mol/L NaOH after degassing of the sample and expressed as g L^{-1} tartaric acid.

2.3. FTIR instruments

In this study three commercially available FTIR instruments using different techniques for spectra acquisition were compared.

The first instrument was a FT2 Winescan™ (Foss electric, Hilleroed, DK). With this instrument spectra were recorded in a range from 926 to 5012 cm^{-1} with a spectral resolution of 14 cm^{-1}. Spectra were recorded at a sample temperature of 40 °C. Measurements were carried out in transmission at a defined optical pathlength of 37 μm using a CaF_2 cuvette. Sampling was conducted with an autosampler, using about 30 ml of sample for a double measurement including preflushing of the system.

The correction for background effects like water vapor in the optical pathway of the instrument is usually done by measuring a standard before the sample measurement. The sample transmittance spectrum is then divided by the background transmittance spectrum obtained in the standard measurement. Background measurements were taken against double distilled water. Background measurements were taken every 20 min and the system was backflushed automatically every 20 min or after 15 samples.

Table 2
Analytical characterisation of the calibration and validation data set.

Parameter	Calibration					Validation				
	Mean	Standard deviation	Min	Max	*n*	Mean	Standard deviation	Min	Max	*n*
Acetic acid (g L^{-1})	0.43	0.13	0.14	0.82	107	0.43	0.10	0.21	0.65	57
Citric acid (g L^{-1})	0.20	0.16	0.01	0.77	96	0.20	0.14	0.02	0.64	57
Relative Density (20 °C/20 °C)	0.9957	0.0048	0.9897	1.0187	108	0.9962	0.0051	0.9907	1.0208	58
Ethanol (g L^{-1})	98.4	9.0	63.2	116.1	108	98.4	8.8	69.2	113.0	56
Fermentable sugars (g L^{-1})	5.6	9.7	0.2	54.2	108	6.6	11.4	0.2	62.7	57
Fructose (g L^{-1})	3.0	5.2	0.1	29.7	108	3.5	6.2	0.1	34.5	57
Glucose (g L^{-1})	2.5	4.5	0.1	24.5	108	3.1	5.3	0.1	28.2	57
Glycerol (g L^{-1})	7.9	1.8	4.4	12.1	108	7.9	1.7	5.5	11.6	57
Lactic acid (g L^{-1})	1.3	0.8	0.1	3.6	107	1.2	0.5	0.1	2.8	57
Malic acid (g L^{-1})	0.9	1.3	0.0	6.0	107	0.9	1.1	0.1	3.8	57
pH	3.5	0.2	2.9	3.8	108	3.4	0.2	3.1	3.8	56
Total acidity (g L^{-1})	5.4	0.8	4.0	8.0	108	5.3	0.6	3.9	7.0	56

In this instrument, the interferometer system is totally encapsulated to minimize the disturbance by water vapor and other gases like CO_2 in the optical pathway.

The second instrument was an OenoFoss™ (Foss electric, Hilleroed, Denmark). Spectra were recorded in a range from 936 to 5995 cm^{-1} with a spectral resolution of 14 cm^{-1}. Measurements were taken in transmission with a cuvette with variable optical pathlength. The optical pathlength in these cuvettes is adjusted mechanically. First, spectra were recorded at an optical pathlength of 43 μm (240 scans). Then the optical pathway was adjusted mechanically to 18 μm and 80 scans were recorded. The scans at 18 μm optical pathlength were then used as background measurements analogous to the background measurement against water in the other instruments used in this study. To correct for mechanical uncertainties in the adjustment of the optical pathlength, absorbance spectra were then corrected by a SNV (Standard normal variate) procedure. As a temperature adjustment is not possible with this instrument, samples were analyzed at room temperature. The sample was pipetted directly into the cuvette (400 μl) and cleaning of the cuvette was performed manually with a paper towel.

The third instrument was an Alpha P™ (Bruker, Ettlingen, Germany). Spectra were recorded in a range from 375 to 4000 cm^{-1} and spectral resolution was set to 8 cm^{-1}. Measurements were carried out in single-bounce attenuated total reflectance (SB-ATR) on a diamond ATR-crystal. Spectra were recorded at 40 °C sample temperature with background measurements against ultra-pure water. The ATR crystal is covered with a flow-through cell, facilitating sample injection. Sample injection was performed with a syringe, using 5 ml of sample. Penetration depth into the sample depends on wavelength and the refractive index of the sample in ATR measurements (Cocciardi et al., 2005; Gottwald & Wachter, 1997) and was about 2 μm in the fingerprint region (900–1500 cm^{-1}). For both background and sample measurements 64 scans were recorded. One background measurement was taken before each sample measurement.

2.4. Data processing and spectra pretreatment

Data of all three spectrometers were exported to MATLAB™ (The Mathworks Inc., Natick, Mass., USA) using in-house conversion routines programmed in MATLAB. All calibrations and subset selections were computed using MATLAB PLS Toolbox (Eigenvector Research Inc., Eaglerock, USA). Before computing variable selections and calibrations, water bands and spectral regions with a low signal to noise ratio were removed from the spectra. Three spectral ranges were selected for calibration for each instrument. The pretreated spectra contained data points from 830–1608 cm^{-1}, 1608–1771 cm^{-1} and 2640–3007 cm^{-1} for the SB-ATR instrument, 953–1608 cm^{-1}, 1689–1775 cm^{-1} and 2773–3005 cm^{-1} for the transmission instrument with defined pathlength and 936–1605 cm^{-1}, 1859–2325 cm^{-1} and 2835–3020 cm^{-1} for the transmission instrument with variable pathlength. Mean centering was applied to all spectra before performing variable subset selection and calibration.

2.5. Variable selection

For the Full PLS, all data points in the pretreated spectra were used without performing additional variable selections.

Forward iPLS (Abrahamsson, Johansson, Sparen, & Lindgren, 2003; Christensen, Norgaard, Heimdal, Pedersen, & Engelsen, 2004; Norgaard et al., 2000) was performed using the iPLS option in PLS Toolbox. In iPLS, the spectrum is split into intervals of equal size. PLS regressions are performed for every interval. Once all intervals have been cross validated, the interval with the lowest RMSECV is selected as the first interval. A second set of PLS regressions is then performed using the first interval in combination with any remaining interval. Stepwise, intervals are added until the RMSECV does not improve anymore. In this study an interval size of 10 variables and a maximum number of 15 LVs in the PLS regression were used. Overlapping intervals were not allowed.

Genetic Algorithm (GA) variable selection was performed using the settings recommended by Leardi (2000) with the modification that window size was set to one variable.

Principal variable PLS (PVPLS) was performed using an in house MATLAB routine which was programmed after the subset selection procedure suggested by Höskuldsson (2001). The number of selected variables was limited to 30.

2.6. Calibration and validation

All calibrations were calculated with the SIMPLS algorithm employed in the PLS toolbox. For calibration development, cross validation was used. Data splitting in cross validation was performed by the venetian blinds method, splitting the data set in 10 parts. In order to obtain reproducible numbers of latent variables to be included in a calibration, the suggestion made by the PLS Toolbox software was followed. The suggestions made by the software are calculated by the ''choosecomp''-algorithm (Eigenvector Inc., 2010). The default settings for the calculation of the suggestions remained unmodified.

Outliers were detected using *Q* Residuals, Hotelling T^2, leverage and deviation in prediction. Spectra with high *Q* residuals and Hotelling T^2 values were considered untypical spectra. They were only eliminated if they showed high leverages or deviation and their elimination improved the model significantly. Values with high deviation in prediction which did not show high *Q* or T^2 values were considered to be reference value outliers and eliminated. After calibration development, all methods were saved and the validation data set was analyzed with the methods.

2.7. Statistics

All statistics were computed in MatLab PLS Toolbox (Eigenvector Research Inc., Eaglerock, USA) and Statistics Toolbox. Primary result obtained from the software was RMSEP. This value is termed $RMSEP_{raw}$. To correct the calibration results for reference method errors, the method proposed by Faber and Kowalski (1997) was used. As suggested in their work, 30 degrees of freedom were assumed for the variance of the reference methods and the overcorrection risk α was set to 5%. Reference method errors were derived from the sources stated in 2.1. Standard deviation was calculated by dividing the repeatability by a factor 2.8 (95% confidence). The corrected $RMSEP_{raw}$ is termed $RMSEP_{true}$. Further, if BIAS in the validation results was significant, the RMSEP was corrected for bias using Eq. (1).

(1), $RMSEP_{korr}^2 = RMSEP_{true}^2 + BIAS^2$

The $RMSEP_{true}$ equals the standard error of prediction (SEP) in the case that any further sources of bias can be ruled out. For the comparison of the instruments, Williams and Sobering (1993) introduced the statistic RPD (ratio of prediction to standard deviation). In this study we used the RPD of the validation and the mean number of latent variables for the evaluation of instrument and variable selection performance. The RPD of validation is calculated as the standard deviation of the validation data set divided by $RMSEP_{true}$ (Kim, Himmelsbach, & Kays, 2007). An RPD value of <3.0 is considered ''poor'', while spectroscopic calibrations with an RPD of >5 are considered ''good'' and with an RPD of >8.0 ''excellent'' (Williams & Norris, 2001).

2.8. Spectral standard deviation

To determine spectral standard deviation, a set of five wines (two red wines, one dry white wine, one sweet white wine, one rosé wine) was measured five times on all three instruments. The relative spectral standard deviation was calculated as the mean standard deviation of all five samples divided by the maximum minus the minimum absorption of the data set in the fingerprint region.

3. Results and discussion

3.1. Comparison of spectra

Absorption of the spectra of the ATR and transmission instruments was proportional to the optical pathlength at the specific wavenumber, e.g. the transmission instruments showed an absorption about 19-times higher than the ATR instrument at the peak absorption at 1045 cm^{-1} (C–O stretch vibration). The transmission – variable pathlength spectra showed a very different behavior due to two reasons: The background measurement was done in the sample itself and the spectrum had been SNV-corrected already by a built-in spectrometer routine. In the SB-ATR spectra there is no total absorption at H–O–H stretching and bending vibrations (water bands). The generally lower absorbance in SB-ATR spectra makes these spectral regions accessible for calibration. One drawback of the SB-ATR instrument is the absorption of the diamond ATR crystal at 1900–2300 cm^{-1} (Walker, 1979).

3.2. Spectral standard deviation

The relative spectral standard deviation was highest in the transmission instrument with variable pathlength cuvette and lowest in the transmission instrument with defined pathlength cuvette. The ATR instrument showed a lower spectral standard deviation than the variable pathlength instrument. However, the standard deviation in the ATR instrument was clearly due to noise, while spectral standard deviation in the variable pathlength instrument was probably due to offset. The offset may have been introduced by deviations in the mechanical adjustment of the optical pathlength, which could not be entirely eliminated by the automatic SNV-correction performed in this instrument (Fig. 1).

Plotting the relative standard deviation over the fingerprint range showed a clear maximum of the standard deviation at 1045 cm^{-1} (C–O stretching vibration) in both transmission measurements. This may be explained by the fact that radiation at this wavenumber is almost totally absorbed by the C–O bonds at the pathlengths used in the transmission instruments, and very little light reaches the detector, which is leading to inaccurate measurements.

3.3. Instrument comparison

An overview of all calibration results obtained in this work and expressed as RPD and RMSEP is given in Table 3 for the respective instruments. The correction of RMSEP for the measurement errors of the reference analysis did not improve the RMSEP drastically. The maximum improvement of RMSEP was obtained with the parameter lactic acid, which was about 2%. As a source of error, the reference analysis therefore seems to play only a minor role in FTIR spectroscopy for wine analysis.

3.3.1. Ethanol

All instruments used in this study showed a very good capacity for the analysis of ethanol in wine (RPD 5.8–9.1). Best performance was achieved by the transmission instrument with fixed pathlength (mean RPD 8.5, mean LVs 3.5), followed by the ATR instrument (mean RPD 8.1, mean LVs 3.5) and the transmission instrument with variable pathlength (mean RPD 7.1, mean LVs 5). These results are comparable or better than the results available in literature for SB-ATR and transmission instruments with defined pathlength (Cocciardi et al., 2005; Cozzolino et al., 2011; Patz et al., 2004). Ethanol, as the quantitatively most important wine component after water, is the main contributor to the C–O stretch peak at 1045 cm^{-1}, which showed the highest standard deviation in the spectra of the transmission instrument with variable pathlength. Parameters for which the inclusion of the C–O stretching vibration peak at 1045 cm^{-1} was unavoidable in calibration (ethanol and relative density, which showed the highest loadings at this peak) were easy and precise to calibrate on the ATR instrument and the transmission instrument with defined pathlength, while the calibrations obtained with the transmission instrument with variable pathlength were less precise and/or robust, especially with the parameter relative density (3.3.6).

While the FullPLS model showed the highest RPD with the transmission instrument with fixed pathlength, PVPLS shows the highest RPD values with the transmission instrument with variable pathlength and the ATR instrument.

3.3.2. Sugars

All three instruments used in this study showed an excellent capacity for the analysis of sugars (RPD 3.8-20.1). Fermentable sugars and glucose calibrations were most precise for the transmission instrument with defined pathlength (mean RPD 13.3, mean LVs 7 for fermentable sugars and mean RPD 9.4, mean LVs 5 for glucose). These results are comparable to those obtained in earlier studies with a similar instrument (Gishen & Holdstock, 2000; Patz et al., 2004). The most accurate fructose calibrations were obtained with the transmission instrument with variable pathlength (mean RPD 11.5, mean LVs 6). Although showing comparable validation results for all three sugar parameters, the models for the ATR instrument were the least accurate. However, the results are comparable to

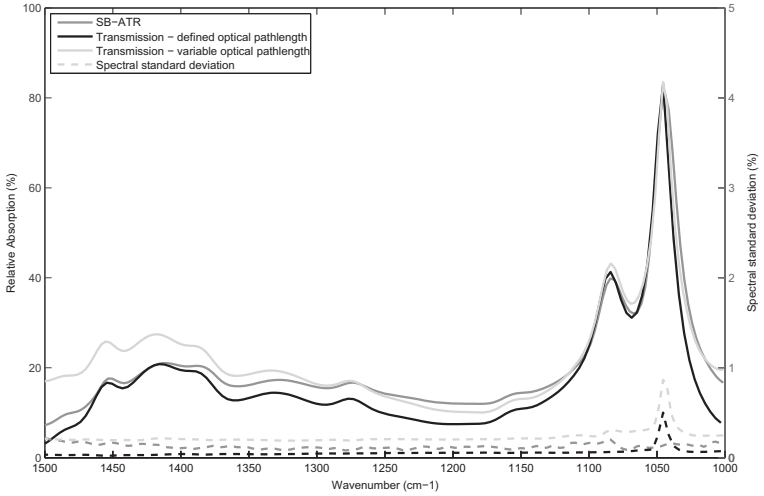

Fig. 1. Relative absorption and spectral standard deviation of the ATR instrument (dark grey) and the transmission instruments with variable (light grey) and defined pathlength (black) cuvettes. Spectral standard deviations are plotted in interrupted lines.

data available in literature (Cocciardi et al., 2005; Cozzolino et al., 2011). It is clearly visible that the parameter with the highest standard deviation in our data set, the fermentable sugars, also yielded the highest RPD of all sugars, while the sugar parameter with the lowest standard deviation and concentration, glucose, also yielded the lowest RPD. In terms of variable selection algorithms, the best models were obtained with GAPLS on the transmission instrument with defined pathlength, while iPLS produced the best models on the other two instruments.

3.3.3. Glycerol

Glycerol is present in wine in concentrations ranging from 5 to 20 g L^{-1} (Ribereau-Gayon, Glories, Maujean, & Dubourdieu, 2006a). In our data set, the average glycerol concentration is much higher than the average concentration of sugars, but the standard deviation is much lower than the standard deviation of even the individual sugars. This may explain that the RPD values for glycerol calibrations are on average much lower than RPD values for sugar calibrations. The most accurate glycerol calibrations were obtained with the transmission-variable pathlength instrument (mean RPD 4.5, mean LVs 8.5). Mean RPD for the transmission-defined pathlength instrument was 4.4 with 7.5 latent variables, while the ATR instrument calibrations showed RPD values from 2.72 to 3.16 (mean 2.9, mean LVs 6.3). RPD values under 3 are considered as poor (Williams & Norris, 2001). GAPLS showed the highest RPD values for the transmission instruments, while the best ATR calibration was obtained with PVPLS (RPD 3.16, 6 LVs).

3.3.4. Lactic acid and malic acid

Lactic acid is produced from malic acid in red and certain white wines during malolactic fermentation (MLF). If a wine does not undergo MLF, lactic acid is only present in small amounts. If a wine does undergo MLF, malic acid is usually completely metabolized to lactic acid and CO_2 (Ribereau-Gayon, Lonvaud, Dubourdieu, & Doneche, 2006b). Wines which don't undergo MLF are usually supposed to have a fresh taste and therefore will have a higher (natural or added) acid content. A typical wine data set (containing about half MLF and half non MLF wines) will therefore show higher maximum malic acid than lactic acid. Also, standard deviation of malic acid will be higher than the one of lactic acid, as malic acid values will be either high or close to zero. Therefore, generally, higher malic acid RPD values can be expected in FTIR calibrations for wine analysis.

This assumption was confirmed in our study. On all three instruments, RPD values were lower for lactic acid (RPD 1.7–4.2) as compared to malic acid calibrations (RPD 2.1–6.0). The most accurate malic and lactic acid calibrations averaged over 4 variable selection methods were obtained with the transmission – variable pathlength instrument, (mean RPD 4.3 and 3.0 for malic and lactic acid, respectively). For the analysis of malic and lactic acid, the transmission cell instrument with defined pathlength showed slightly less precise validation results even though the technical standard of this instrument is considerably higher due to the encapsulation of the interferometer system. There is no temperature control for the sample in the variable cuvette pathlength instrument, so temperature differences may further introduce differences in absorption to the spectra. Further mechanical adjustment of the optical pathlength with following SNV-correction did introduce a higher spectral standard deviation due to spectral offset (Fig. 1).

An explanation for the better performance of the transmission – variable pathlength instrument may be that malic and lactic acid mainly absorb mid-infrared radiation in spectral regions with less pronounced absorption and not in the peak region around 1045 cm^{-1} (Vonach, Lendl, & Kellner, 1998). The larger pathlength (43 μm) of the transmission instrument with variable cuvette pathlength may lead to a higher signal to noise ratio in the regions where the absorption peaks of the organic acids lie. Finally, effects of spectral offset can be eliminated in the PLS calibration which confers the use of a higher number of latent variables to model the differences in absorption, as seen in the calibrations (Table 3).

None of the calibrations for any of the acids with the ATR instrument showed an RPD value over 3.0, which would be considered as a "fair" calibration. The low signal-to-noise ratio of the ATR

Table 3
Results of all calibrations performed with the ATR instrument and the transmission instruments with defined and variable pathlength.

Instrument	Parameter	FullPLS			GA			iPLS			PVPLS		
		LVs	RPD	$RMSEP_{raw}$	LVs	RPD	$RMSEP_{raw}$	LVs	RPD	$RMSEP_{raw}$	LVs	RPD	$RMSEP_{raw}$
SB-ATR	Relative Density (20 °C/ 20 °C)	5	13.8	0.00038	3	14.1	0.00043	2	12.1	0.00045	4	11.6	0.00046
	Ethanol (g L^{-1})	3	8.5	1.49	5	7.2	1.25	3	7.7	1.55	3	8.9	1.58
	Fermentable sugars (g L^{-1})	6	9.2	1.27	5	8.2	1.44	4	15.8	0.73	5	9.4	1.29
	Fructose (g L^{-1})	7	9.3	0.70	5	9.1	0.69	4	8.5	0.74	5	10.7	0.61
	Glucose (g L^{-1})	6	7.0	0.75	6	6.0	0.87	4	7.6	0.71	4	6.7	0.79
	Glycerol (g L^{-1})	6	2.7	0.65	7	2.8	0.60	6	2.9	0.58	6	3.2	0.57
	Lactic acid (g L^{-1})	6	1.8	0.31	8	1.8	0.32	6	2.0	0.28	6	1.7	0.33
	Malic acid (g L^{-1})	8	3.0	0.36	7	2.1	0.51	5	2.1	0.51	7	2.9	0.37
	pH	7	1.9	0.08	7	1.7	0.10	4	2.0	0.08	3	2.1	0.08
	Total acidity (g L^{-1})	6	2.1	0.29	7	1.7	0.36	5	1.9	0.36	8	2.5	0.25
Transmission -defined pathlength	Relative Density (20 °C/ 20 °C)	4	17.0	0.00031	2	16.7	0.00032	4	15.6	0.00036	4	15.0	0.00036
	Ethanol (g L^{-1})	3	8.8	1.02	3	8.5	1.09	5	8.4	1.07	3	8.1	1.12
	Fermentable sugars (g L^{-1})	6	10.5	1.09	8	20.1	0.63	7	7.8	1.55	7	14.9	0.80
	Fructose (g L^{-1})	4	9.1	0.68	6	12.5	0.53	4	7.4	0.86	7	13.5	0.51
	Glucose (g L^{-1})	5	7.2	0.74	4	9.7	0.55	5	11.4	0.50	6	9.2	0.57
	Glycerol (g L^{-1})	8	4.4	0.41	8	4.8	0.39	6	4.7	0.36	8	3.5	0.49
	Lactic acid (g L^{-1})	8	2.4	0.22	9	4.2	0.15	7	1.6	0.34	9	2.3	0.23
	Malic acid (g L^{-1})	7	3.7	0.29	5	3.1	0.35	8	5.4	0.20	8	3.2	0.34
	pH	6	2.1	0.08	7	2.7	0.07	4	2.5	0.07	5	1.9	0.10
	Total acidity (g L^{-1})	5	3.2	0.19	4	4.0	0.18	6	4.3	0.17	8	4.2	0.19
Transmission - variable pathlength	Relative Density (20 °C/ 20 °C)	6	13.0	0.00039	3	5.6	0.00104	7	11.6	0.00044	4	7.4	0.00077
	Ethanol (g L^{-1})	4	5.8	1.52	6	6.9	1.41	6	6.7	1.32	4	9.1	1.34
	Fermentable sugars (g L^{-1})	7	10.2	1.12	7	15.6	0.74	7	12.8	0.90	6	11.1	1.05
	Fructose (g L^{-1})	7	10.5	0.59	3	6.1	1.08	7	17.2	0.38	7	12.3	0.51
	Glucose (g L^{-1})	7	8.6	0.62	3	3.8	1.41	6	8.0	0.67	6	7.8	0.68
	Glycerol (g L^{-1})	9	4.1	0.42	8	5.2	0.35	8	4.6	0.41	9	4.1	0.42
	Lactic acid (g L^{-1})	10	2.9	0.20	9	3.5	0.17	7	2.8	0.20	9	3.1	0.18
	Malic acid (g L^{-1})	9	4.0	0.30	9	6.0	0.19	9	2.7	0.40	11	4.1	0.27
	pH	9	2.6	0.06	8	2.7	0.06	7	2.3	0.07	8	2.1	0.08
	Total acidity (g L^{-1})	9	2.9	0.21	8	3.9	0.15	5	2.6	0.23	11	2.8	0.22

instrument in the spectral regions in which parameters like organic acids absorb MIR-radiation makes the instrument less precise for quantitatively minor wine parameters as compared to the other two instruments. In a study on apricots, using multiple (6-fold) bounce attenuated total reflection on a ZnSe crystal, Bureau el al. (2009) obtained very good calibration results for various organic acids contained in this fruit. Therefore it seems reasonably clear, that for an analysis of multiple parameters including the organic acids, a higher absorption is crucial to improve the signal-to-noise ratio in regions where the organic acids show their main absorption.

The most accurate calibrations on the ATR instrument were obtained with FullPLS for malic acid and iPLS for lactic acid. With both transmission instruments, the best calibration for both acids were obtained with GAPLS, with the exception of the malic acid calibration with the transmission – defined pathlength instrument. This calibration model seemed to be underfit, using only five LVs, as compared to at least seven LVs used in the calibrations with the other three variable selection methods.

3.3.5. Total acidity

Total acidity is a summary parameter for all organic and inorganic acids in wine, and is expressed as tartaric acid by convention. As total acidity is determined by titration to a predefined pH value (7.0), not only the concentration of the acids, but also the acidobasic buffer capacity play a mayor role in the final result (Ribereau-Gayon et al., 2006a). This underlying complexity makes the parameter hard to predict spectroscopically by using PLS, and impossible to determine by methods such as science based calibration (SBC), which rely on pure component spectra (Marbach, 2010).

In our study, validation results showed that in most cases models for the prediction of total acid were less accurate than those for the prediction of malic acid, although the mean of total acidity (5.9 g L^{-1}) is generally much higher than the mean of malic acid (0.9 g L^{-1}) in wine. This may be explained to a certain extent by the lower standard deviation of total acidity as compared to malic acid and by the complexity of this parameter. The most accurate calibrations for total acidity were obtained with the transmission – defined pathlength instrument combined with iPLS and PVPLS. However, GAPLS results were of comparable quality, using only four latent variables as compared to six and eight for iPLS and PVPLS, respectively. The GAPLS model for this instrument might therefore have been underfit.

With the transmission – variable pathlength instrument, the only calibration to be considered "fair" was obtained with GAPLS; with the ATR instrument, no suitable calibrations for total acidity were obtained, regardless of the variable selection algorithm (maximum RPD 2.5 with PVPLS). Cozzolino et al. (2011), using the same SB-ATR instrument as in this study, recently published results for the analysis of six parameters in wine, which are in substantial agreement with the results obtained in this study. Using a similar data set and 14 LVs in FullPLS, SEP for total acidity was 0.53 g L^{-1} and RPD was 2.1, almost identical with our validation result (2.08 for FullPLS). Similarly, Cocciardi et al. (2005) compared an SB-ATR to a FTIR instrument equipped with a defined optical pathlength CaF_2 cuvette on a small data set and four parameters. Their results are in general accordance with the results obtained in this study, i.e. SB-ATR showed equal or better performance for the analysis of sugars and ethanol in wine, while validation results with the transmission instrument were more accurate for the analysis of total acidity. This may serve as further proof for our hypothesis that

the potential for the analysis of quantitatively minor compounds in wine is limited with SB-ATR instruments.

3.3.6. Relative density

Relative density is a function of all components in wine, but main influence factors are sugars (with the exception of completely dry wines) and ethanol as these substances are present in the highest concentration (Moreira & Santos, 2004) and show the highest standard deviation. Therefore, the most prominent spectral information available is also the most important information for the calibration of relative density. This was confirmed by viewing the loadings of the first and second principal component for all three instruments in a FullPLS calibration. While the alcohol C–O stretching vibration at 1045 cm^{-1} had high loadings on the first two LVs in the ATR and transmission instrument with defined optical pathlength, loadings on this peak were much lower in the calibration with the transmission instrument with variable optical pathlength.

Mean RPD for relative density were 16.0 (mean LVs: 3.5), 12.9 (mean LVs: 3.5) and 9.4 (mean LVs: 5) for the transmission – defined pathlength, the ATR and the transmission – variable pathlength instrument, respectively. The low number of LVs used for the calibration of relative density confirms that prominent structures in the spectra are used for the calibration. The ATR instrument, showing the lowest spectral standard deviation in the most absorptive wavebands, therefore has an advantage for relative density analysis. The transmission – variable pathlength instrument, displaying the highest spectral standard deviation and the lowest loadings in the highly absorptive wavebands, also showed the highest prediction errors and lowest RPDs. Results for the transmission instrument with defined cuvette pathlength were comparable to those obtained in a previous study (Patz et al., 2004). Cozzolino et al. (2011), using the same SB-ATR instrument as in this study, published validation results with a higher validation error, however this may be attributed to a less precise reference method. The same research group (Shah, Cynkar, Smith, & Cozzolino, 2010), using the same instrument, obtained good PLS results for the analysis of total soluble solids (°Brix) in grape juice, which is highly correlated with relative density, but much worse results for the analysis of parameters present in low concentration, like yeast assimilable nitrogen or phenols.

FullPLS yielded the lowest RMSEP and the highest RPD for all instruments for the parameter relative density. Mean RPD was 14.6 (mean LVs: 5.0), 13.1 (mean LVs: 4.3), 12.1 (mean LVs: 2.7) and 10.8 (mean LVs: 4.0) for FullPLS, iPLS, GA and PVPLS, respectively. As all components present in wine will have an influence on its relative density, it may be concluded that the inclusion of a high number of variables, although making a use of a higher number of latent variables necessary, does improve calibration models for this parameter. Again, the use of FullPLS was linked to a high number of latent variables in the calibration, while the use of GA variable selection produced models of comparable quality with only about half the number of latent variables.

3.3.7. pH

The lowest RPD values of all parameters were obtained in the pH-calibrations. Here, mean RPD values were 2.3 (mean LVs: 5.5), 2.4 (mean LVs: 8) and 1.9 (mean LVs: 5.3) for the transmission instruments with defined and variable pathlength, and the ATR instrument, respectively. The high number of LVs already gives a hint that the structures which are less dominant in the spectra need to be included in a reliable pH calibration. pH as an electrochemical and log-scaled parameter can – by a spectroscopic method – only be determined indirectly. Regarding the variable selection, mean RPD was 2.2 (mean LVs: 7.3), 2.4 (mean LVs: 7.3), 2.3 (mean LVs: 5) and 2.0 (mean LVs: 5.3) for FullPLS, GA, iPLS and PVPLS, respectively. As pH calibration relies on delicate structures in the spectrum, it is not surprising that GA-PLS yielded the best results for this parameter, although using a high number of latent variables.

3.3.8. Comparison of variable selection algorithms

Compared to FullPLS, the number of variables (spectral dimension) was reduced by about 92.5% by GA-PLS and PV-PLS and 75% by forward iPLS. Similar to the results presented in a study comparing variable selection algorithms, but on a different matrix using a NIR instrument (Balabin & Smirnov, 2011), only a small improvement as compared to FullPLS was observed in calibration error when using forward iPLS and PVPLS. With a few exceptions, iPLS results were superior to PVPLS, probably due to maintaining continuous blocks of variables (Norgaard et al., 2000). The inclusion of up to 459 variables in FullPLS will make models less robust, which is reflected by the higher number of latent variables used in most FullPLS calibrations.

Genetic algorithms proved superior to the other two variable selection methods and to FullPLS when used with the two transmission instruments. With the transmission instruments GAPLS was yielding calibrations with higher RPD, lower RMSEP and a smaller number of LVs in calibration. One exception is the parameter relative density, where RPD increased with the number of variables used in the model and FullPLS showed the best results.

However, using GAPLS in combination with the ATR instrument did neither improve calibration results nor the number of latent variables as compared to FullPLS. The risk of overfitting models to the calibration data increases with the inclusion of noisy variables in the GA data set. This may have been the case with the ATR instrument in our study. Noise can be reduced by choosing larger windows in the GA settings. However, by choosing large windows, spectral information might be lost. In this study, overfitting with GA variable selection did not occur in the two transmission-type instruments, but the ratio of cross validation error to calibration error was much smaller in the ATR instrument as compared to the other instruments and variable selection algorithms. This is a sign that, by including noisy variables in the selection, the genetic algorithm had overfit the PLS-models to the calibration data set.

4. Conclusion

The main source of the differences observed in calibration results is the cuvette pathlength or penetration depth into the sample, and not the measurement technique itself. Results for the respective instruments depend strongly on the parameters analyzed. For parameters present in low concentration (e.g. acids), transmission instruments with large pathlength yield better results than an SB-ATR instrument, which allows only semiquantitative determination of these compounds. For relative density, ethanol and sugars the results obtained with an SB-ATR instrument are comparable to or better than those obtained with transmission instruments with long pathlengths. The use of multiple bounce ATR instruments in wine analysis is a promising alternative to single bounce ATR instruments. A SB-ATR instrument is suitable for routine wine analysis, however its main advantage, the low penetration depth into the sample, makes it better suited for highly absorptive or cloudy samples like grape juice, must under fermentation, spirits or concentrates. Genetic algorithm variable selection can make PLS models more robust and precise compared to iPLS, PVPLS and full spectrum PLS, however a risk of overfitting remains when noisy variables are present in the data set.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2013.06.120.

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Chapter 3

Impact of light exposure on fruit composition of white 'Riesling' grape berries (*Vitis vinifera* L.)[2]

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Impact of light exposure on fruit composition of white 'Riesling' grape berries (*Vitis vinifera* L.)

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Summary

Microclimate and irradiation have long been known to influence winegrape (*Vitis vinifera*) quality. However, microclimate influence on white grape quality has remained understudied, as most research efforts have focused on red varieties and their anthocyanin content. In this study, we investigated microclimatic effects on the phenolic and amino acid composition of white 'Riesling' grapes using bunch shading and leaf removal to manipulate grape microclimate. Both treatments were applied directly after fruit set (modified E-L 27; (Coombe 1995)) as well as at the onset of veraison (E-L 34), and compared to a non-manipulated control. The concentration of malic acid, amino acids and total nitrogen were decreased by illumination during the berry growth, while content and concentration of phenolics were significantly increased by illumination. Strong negative correlations were observed between accumulation of amino acids and flavonols. Although accumulation of flavonols occurred throughout berry development, the most important phase of accumulation was post-veraison.

K e y w o r d s : Berry composition; leaf removal; light exposure; phenolics.

Introduction

Leaf removal in the bunchzone is one of the most powerful tools for grape producers to influence grape composition and soundness (Smart and Robinson 1991). Modern viticultural management strategies make use of techniques like early leaf removal in order to improve canopy microclimate and grape composition, but also because the removal of the leaves as assimilate source at an early stage of development slows berry growth and leads to a lower susceptibility to bunch rot (Poni *et al.* 2006). The reaction of vines to leaf removal depends on leaf removal severity and timing as well as on the grape variety (Molitor *et al.* 2011, Kotseridis *et al.* 2012, Nicolosi *et al.* 2012). It seems evident that only severe reduction of leaf area before or shortly after flowering will reduce berry size and yield (Ollat and Gaudillere 1998, Poni *et al.* 2006). By applying severe leaf removal during an early stage of berry development, light absorption by the growing berries and, correspondingly, berry temperature are increased. Light interception by grapes has shown to affect the concentration of berry volatiles (Reynolds and Wardle 1989, Bureau *et al.* 2000), phenolics (Price *et al.* 1995, Downey *et al.* 2006) or amino acids (Schultz *et al.* 1998), as well as berry growth (Dokoozlian and Kliewer 1996).

Phenolics display important health benefits and contribute to the sensory perception of foods and beverages (Lesschaeve and Noble 2005). In red wine, phenolics contribute positively to color, taste and shelf-life, while they lead to undesired browning reactions in white wine (Singleton 1987). Furthermore, phenolics are regarded as negative contributors to the sensory properties of white wines associated with bitterness and adringency (Singleton *et al*. 1975, Arnold *et al.* 1980). Phenolics can complex with proteins in wines, leading to haze formation (Ferreira *et al*. 2001).

Amino acids are essential for yeast nutrition and therefore influence the successful fermentation of grape juice (Bell and Henschke 2005). Furthermore, they play a role as wine aroma precursors (Pripis-Nicolau *et al*. 2000, Tominaga *et al.* 1998). An oversupply of amino acids, especially arginine, may lead to the formation of the cancerogenic ethyl carbamate in wine (Ough *et al.* 1988). Little data are available on the effect of light exposure on the composition and growth of white winegrapes, although practices like leaf removal have become increasingly popular in white winegrape production (Poni *et al.* 2006).

The aim of this study was to determine the effects of different irradiation regimes applied at different developmental stages on the accumulation of phenolic compounds, amino acids, sugars and organic acids of white 'Riesling' grape berries.

Material and Methods

E x p e r i m e n t a l s i t e : Field experiments were conducted in the 2011 and 2012 growing season using 'Riesling' (clone Gm 198-25; grafted to rootstock 'SO4 Gm47') in an established vineyard located close to Geisenheim, Germany (49° 59’20” N; 7° 55’56”E). Vines were cane pruned and trained to a vertical shoot positioning (VSP)-type canopy system in a north-south row orientation (Row azimut 164°). Row and vine spacing was 2.10 and 1.05 m, respectively. In order to obtain a homogenous canopy, the shoot number was adjusted to ten shoots per vine.

F i e l d t r i a l : A field trial was established in randomized complete block design with three replicates. Be-

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tween each of the blocks, one row of vines was left as a buffer row. Each replicate consisted of four vines. Two treatments were applied: One artificial shading treatment and one leaf removal treatment. In the artificial shading treatment, the effect of light on the grapes was excluded by sheltering whole bunches in boxes made of tetra brick foil, as described by Downey *et al.* (2004). The boxes remained on the clusters from the point of treatment application until harvest. In the leaf removal treatment, all leaves and lateral shoots providing shade to the grapes in the bunch zone were removed. Regrowth in the bunch zone was removed at three-week intervals. The trial conducted in 2011 consisted of two separate experiments on leaf removal and shading. Each experiment had a separate control and both were conducted in randomized complete block design with three replicates for the leaf removal and five replicates for the shading trial. Leaf removal was applied 14 d after flowering ([DAF], 12.06.2011), and shading was applied 33 DAF (01.07.2011). The shading trial was harvested 111 DAF (17.09.2011), the leaf removal trial 114 DAF (20.09.2011).

In the 2012 trial, leaf removal and shading treatments were applied directly after fruit set (E-L 27; 02 July 2012; 16 DAF) and at veraison (E-L 34; 11 August 2012; 57 DAF). Bunches sheltered in the boxes cannot be reached by pesticide spraying. Therefore, the boxes were opened at night and sprayed manually on the same days the bunchzone spraying was applied.

Sampling: To ensure that bunches sampled in the experiment were influenced by a similar light climate, only bunches exposed to the western side of the canopy in a height of 80-110 cm above ground were sampled. Sampling took place at the beginning of the trial (07/02/2012, 16 DAF, only berry skin phenolics), at veraison (08/10/2012, 57 DAF) and at harvest (10/16/2012, 123 DAF). In the 2011 trial, sampling was only conducted at harvest. Sample size for the analysis of berry skin phenols was 20 berries per replicate from four bunches of different vines (five berries per bunch) at veraison and harvest, and 50 berries from four bunches at the first sampling date. The berries were cut off with their pedicel and stored immediately under CO_2 atmosphere and frozen at -20 °C. Berries were peeled whilst frozen. Skins were then freeze dried, ground and stored in an exsiccator until analysis. Skin water content was calculated as (skin fresh weight - skin dry weight) / skin fresh weight. Sample size for the analysis of grape juice parameters was 100 randomly selected berries per replicate from four bunches (25 berries per bunch). The samples were pressed for 5 minutes and filtered through a 16 µ Munktell 33/N folded filter (90 g m^{-2}; Ahlstrom, Helsinki, Finland) prior to analysis.

Microclimatic measurements: Temperature and humidity were monitored by placing three temperature probes (LASCAR, UK) inside the boxes and in the surrounding canopy respectively for the duration of the experiment. Incident radiation in the boxes was measured by inserting three LI-190 SA50 Quantum Sensors (Li-Cor, Lincoln, USA) connected to a LI-1400 data logger inside the boxes and on the western side of the canopy. These data were compared to ambient photon flux density measured by a weather station of the German Meteorological Service approximately 1 km from the experimental vineyard. Bunch surface temperatures were measured by infrared thermography (H2640, NEC Avio Infrared Technologies, Tokyo, Japan) on three days (17.08.; 30.08.; 31.08.2011). Measurements were taken in the morning (8:00-9:00), at noon (12:30-13:30) and in the afternoon (15:30-16:30) on exposed bunches, bunches sheltered in boxes, and bunches under one and two leaf layers on the western side of the canopy. Mean temperature for control bunches was calculated from point quadrat data and bunch temperatures. Point quadrat analysis (Smart and Robinson 1991) with three replicates of 50 insertions each was utilized to describe canopy conditions at veraison in the central bunch zone. Spacing between insertions was 20 cm.

Analytical approaches: Grape juice was analyzed for pH, titratable acidity, malic acid, relative density and the concentration of glucose and fructose by Fourier-Transform Infrared Spectroscopy using an in-house calibration on a FT2 Winescan Instrument (Foss Electric, Denmark). Berry amino acids (only 2012) were analyzed with an amino acid analyzer S433 (Sykam, Eresing, Germany). Chromatographic separation was achieved on a 4.6 x 150 mm LCA K 07/Li cation-exchange column (Sykam) with post-column ninhydrin derivatisation and photometric detection at 570 and 440 nm for primary and secondary amino acids. α-Amino acid concentration was also analyzed by the N-OPA method, following the protocol of Dukes and Butzke (1998).

For HPLC analysis of phenolics, phenolic compounds were extracted from the freeze dried grape skin powder in acidified acetonitrile under SO_2 protection followed by vacuum distillation of the extracts. The extracts were analyzed by an ACCELA HPLC/DAD system coupled to a LXQ mass spectrometer (ThermoFisher, Dreieich, Germany). Chromatographic separation was achieved on a 150 x 2 mm i.d., 3 µm Luna 3u C18 100A column (Phenomenex, Aschaffenburg, Germany) protected with a guard column of the same material. Injection volume was 3 µL, at a flow rate of 250 $\mu L \cdot min^{-1}$. Elution conditions were: solvent A was 2 % acetic acid; solvent B was acetonitrile/Water/acetic acid (50:50:0,5; v/v/v). Gradient elution was applied: 0-20 min from 96-50 % solvent A, 4-50 % solvent B, 20-23.1 min to 100 % B; washing with 100 % B for 2 min before re-equilibrating the column. Detection wavelengths were 280 nm for flavanols, 320 nm for phenolcarbonic acids and 360 nm for flavonols. The following mass spec conditions were used: ESI source voltage -3.00 kV during negative and +5.00 kV during positive ionization mode; capillary temperature 275 °C; collision energy for MS^n-experiments 35 % (arbitrary units). Peak identification was based on a combination of HPLC retention time and UV spectra as well as mass spectral data. Quantification was carried out using peak areas from external calibration curves. A table containing all standard sources is presented as supplemental Table. Where no standards were available, substances were quantified using the calibration for the closest phenolic relatives (caftaric acid as caffeic acid; fertaric acid as ferulic acid, coutaric acid and p-CGT as coumaric acid). Total nitrogen in grape juice and grape

skin powder was analyzed by a modified Kjeldahl-method with ammonia determination by flow injection analysis (FIAstar 5000, Foss, Denmark) with photometric detection at 720 nm (Persson *et al*. 2008).

Data analysis: Experimental results were evaluated using a generalized linear model (GLM) for normally distributed data with treatment, year and sampling date as factors. Post-hoc pairwise comparisons were performed by a Fisher's LSD test. Statistical testing was performed with SPSS 15.0 Software (IBM, Armonk, U.S.). Principal component analysis (PCA) was applied on the harvest data of 2011 and 2012, using autoscaling as data standardisation method. PCA was calculated using MatLab (The Mathworks, Natick, U.S.) software with PLS toolbox (Eigenvector Inc., Eaglerock, U.S.).

Results

Experimental conditions: Point quadrat analysis showed that canopy conditions (number of leaf layers, number of shaded leaves) in the three control blocks and the two experimental years were not significantly different (Tab. 1). The average number of leaf layers in both years was two, with homogenous distribution along the VSP trellis. About 45 % of the clusters were exposed to direct sunlight in the control. Monitoring of PAR showed that bunches sheltered in the boxes were only exposed to approximately 1.6 % of total PAR averaged over a day, compared to 60.3 % for exposed clusters. Temperature and humidity in the boxes were only slightly elevated compared to the canopy environment on a sunny day. This is in accordance with data published by Downey *et al.* (2004), who developed this method of bunch shading. Bunch temperatures were lowest in the shading treatment as direct solar heating of the bunches did not occur. However, the temperature difference to the control bunches was negligible. Exposed clusters showed the highest temperatures, up to 6 °C higher than bunches in boxes and 8 °C higher than bunches shaded by two leaf layers when exposed to peak radiation.

Table 1

Description of cluster environment. †Values ± standard deviation calculated from point quadrat analysis (3 replicates, 50 insertions); ‡Box and leaf removal: mean of nine IR-thermographic measurements on two bunches during 3 days; control: mean of nine measurements on five clusters under different shading levels during three days; § mean of two hot and sunny days (18.-19.08.2012)

		Control	Box	Leaf removal
2011	Leaf layers†	1.99 ± 0.13	n.d.	0
	% exposed clusters†	43.7 ± 12.9	0	100
	% interior leaves†	20.06 ± 1.73	n.d.	0
	Bunch Temperature‡	21.1	20.9	22.7
2012	Leaf layers†	1.98 ± 0.11	n.d.	0
	% exposed clusters†	47.8 ± 18.8	0	100
	% interior leaves†	19.63 ± 0.51	n.d.	0
	PAR (% of Ambient)	n.d.	1.62	60.23
	Air Temperature§	27.21	27.7	n.d.

Grape compounds: In 2011, berry weight was higher than in 2012, but berries showed lower sugar concentration, titratable acidity, malic acid and N-OPA. The effects of shading and leaf removal were similar in both seasons (Tab. 2). Berry weight, total soluble solids and berry skin nitrogen were not affected by the treatments. The pH-value was decreased by leaf removal at E-L 27 and increased by shading at E-L 27 when compared to the control, but remained unaffected when the treatments were applied at veraison. Malic acid was increased by shading, but remained unaffected by leaf removal. N-OPA and total juice nitrogen were strongly affected by the treatments, with leaf removal decreasing and shading increasing the concentration and content of nitrogen compounds in the berries. Although these effects were observed already at veraison in 2012, treatments applied at E-L 27 were not different to treatments applied at E-L 34 when sampled at harvest. The increased concentration of titratable acidity in the shade E-L 34 treatment may be related to the elevated concentration of malic acid observed in this treatment in 2012.

Amino acids: Analysis of the single amino acids in 2012 showed that the amino acid profile at veraison was dominated by glutamic acid, glutamine, aspartic acid and arginine (Tab. 3). Ammonia nitrogen was about twice as abundant as amino acid nitrogen at veraison (data not shown). The ratio of ammonia nitrogen to amino acid nitrogen was significantly elevated in leaf removal bunches compared to shaded bunches. Generally, leaf removal showed a larger effect than shading before veraison, decreasing the amino acid concentration by more than 25 % compared to the control. The fact that NH_4-nitrogen and transport/storage amino acids like glutamine and arginine, as well as glutamic acid, the key amino acid in transamination, were dominating the amino acid profile at veraison corresponds well with this early stage of fruit compositional development.

At harvest, the differences between treatments increased and all amino acids differed significantly between treatments (Tab. 3). However, there was never a difference between the two leaf removal treatments. Surprisingly, the concentration of some amino acids in the late (E-L 34) shading treatment was significantly higher than in the early shading treatment. In general, berries from the shading treatments had a significantly higher concentration of amino acids than control and leaf removal treatments, while control and leaf removal treatments differed significantly only for some amino acids. Amino acid nitrogen was more than four times as abundant as ammonia nitrogen at harvest due to a decrease in ammonia and an increase in amino acid nitrogen concentration. The ratio of ammonia nitrogen to amino acid nitrogen was not influenced by the irradiation regimes at harvest.

The amino acids most strongly affected by the treatments at harvest were arginine, tryptophan, methionine, glutamine and asparagine, while at veraison alanine, asparagine, glutamine and arginine were most severely changed by the treatments. During ripening, the most notable change observed in the amino acid profile was the concentration of proline, which increased 54-fold. Of other quantitatively

Table 2

Ripening parameters of all treatments ± standard deviation at veraison (E-L 34) and harvest, experimental years 2011 and 2012. Leaf removal: all leaves in the bunch zone removed; Shade: Complete shading by covering bunches with boxes impermeable to light. E-L numbers given after the treatment indicate the developmental stage in which the treatment was applied. Titratable acidity is expressed as tartaric acid. Treatment, sampling date and year effects were evaluated using a generalized linear model (GLM). Different letters indicate significant differences for treatments of all sampling dates according to Fisher's LSD test ($p < 0.05$). Year and sampling date differences are given as asterisks on the right hand side of the table. *) $p < 0.05$; **) $p < 0.01$; ***) $p < 0.001$

Date	Harvest 2011		Harvest 2011		Veraison 2012		
Treatment	Leaf removal E-L 27	Control	Shade E-L 29-31	Control	Shade E-L 27	Control	Leaf removal E-L 27
Berry weight [g]	1.54 ± 0.08	1.59 ± 0.01	1.42 ± 0.15	1.49 ± 0.14	0.60 ± 0.01	0.63 ± 0.05	0.68 ± 0.05
TSS [°Brix]	18.29 ± 0.24	17.56 ± 0.65	19.64 ± 1.46	18.79 ± 0.74	6.03 ± 0.21	5.94 ± 0.17	5.82 ± 0.17
TA [g L^{-1}]	8.21 ± 0.08	8.36 ± 0.54	9.71 ± 1.89	8.11 ± 0.99	37.69 ± 0.8	39.47 ± 0.82	39.47 ± 1.46
pH	2.99 ± 0.02	3 ± 0.02	2.98 ± 0.05	3 ± 0.04	n.d.	n.d.	n.d.
Malic acid [g L^{-1}]	1.97 ± 0.21	2.29 ± 0.21	3.29 ± 1.1	1.67 ± 0.39	23.25 ± 0.69	23.00 ± 0.79	22.22 ± 0.66
Total N Juice [mg L^{-1}]	n.d.	n.d.	n.d.	n.d.	257.82 ± 60.07	215.47 ± 7.51	184.31 ± 46.01
Total N Skin [%]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
N-OPA juice [mg L^{-1}]	61.66 ± 7.02	73.33 ± 4.73	98.33 ± 10.69	74.33 ± 13.05	51.00 ± 3.46	50.67 ± 3.21	46.67 ± 8.08

Date	Harvest 2012						
Treatment	Leaf removal E-L 27	Leaf removal E-L 34	Control	Shade E-L 34	Shade E-L 27	Sign year	Sign date
Berry weight [g]	1.21 ± 0.16	1.20 ± 0.12	1.28 ± 0.06	1.19 ± 0.07	1.09 ± 0.17	***	***
TSS [°Brix]	20.77 ± 1.30	20.81 ± 0.17	20.69 ± 0.52	19.35 ± 0.58	21.62 ± 2.11	***	***
TA [g L^{-1}]	10.02 ± 0.09 b	9.66 ± 0.13 b	9.61 ± 0.29 b	11.00 ± 0.91 a	9.70 ± 1.04 b	***	***
pH	2.87 ± 0.06 c	2.92 ± 0.03 bc	2.93 ± 0.02 b	2.96 ± 0.04 bc	3.01 ± 0.04 a	*	n.t.
Malic acid [g L^{-1}]	2.60 ± 0.35 b	2.55 ± 0.15 b	2.94 ± 0.20 b	4.15 ± 0.53 a	3.68 ± 0.63 a	***	***
Total N Juice [mg L^{-1}]	171.00 ± 43.42 c	186.70 ± 10.84 bc	213.03 ± 27.12 b	301.80 ± 15.64 a	294.13 ± 61.42 a	n.t.	-
Total N Skin [%]	0.65 ± 0.05	0.63 ± 0.06	0.61 ± 0.06	0.66 ± 0.02	0.67 ± 0.02	n.t.	n.t.
N-OPA juice [mg L^{-1}]	69.7 ± 6.7 c	74.7 ± 4.0 c	93.3 ± 3.8 b	137.3 ± 9.1 a	131.0 ± 20.9 a	***	***

important amino acids, the concentration of GABA and alanine increased 11.5 and 5.5-fold, respectively. The concentration of aspartatic acid, asparagine and glutamic acid decreased by 79, 32 and 47 % respectively between veraison and harvest. The amount of free amino acids in grape juice correlated strongly with the amount of total nitrogen ($r^2 = 0.72$).

Phenolics: The content of phenols in the berry skin increased from 0.04 mg $berry^{-1}$ at E-L 27 to 0.24 mg $berry^{-1}$ at harvest in the control treatment in 2012. The content of total phenols increased significantly for all sampling dates and in all treatments. In parallel, the water content of the berry skins decreased from 82 % at E-L 27 to 72 % at E-L 34 and to 56 % at harvest (54 % in 2011). No significant differences in skin weight or skin water content were detected between treatments or years.

The content (not shown) and concentration of most skin flavanols and hydroxycinnamic acids rose from E-L 27 to veraison and stagnated or decreased (flavanol concentration) after veraison, with the exception of caftaric, coutaric and fertaric acid, which also increased significantly post-veraison. In contrast, quercetin glycoside content and concentration remained rather stable before veraison, but increased drastically post-veraison. No increase in berry quercetin glycoside content took place in the shading treatments over time, while concentration decreased (Tab. 4). The increase in total phenolic content in these treatments was mainly due to an increasing content of hydroxycinnamic acids, while their concentration stagnated. Before veraison, leaf removal increased the concentration of all detected quercetin glycosides, while concentration in control and shading treatments stagnated. At harvest, leaf removal at E-L 27 showed higher flavonol concentration than leaf removal at E-L 34 due to elevated concentrations of que-3-rutinoside and que-3-glucuronide. Concentration of all flavonols was increased by leaf removal and decreased by shading. Shading or leaf removal effects on non-flavonol-phenols were less clear. The concentration of catechin, fertaric acid, caftaric acid and caffeic acid were increased by leaf removal or decreased by shading, while coutaric acid was increased by shading. No treatment had an effect on total hydroxycinnamic acid or flavanol concentration. Changes in the quercetin glycoside profile were observed throughout fruit development. At berry set and veraison, the main quercetin glycosides present in berry skins were que-3-glucuronide, que-3-glucoside and que-3-rutinoside. These were also the main quercetin glycosides found in shaded bunches in both experimental years. The increase in flavonols after veraison was due to an increase in que-3-glucoside, que-3-galactoside, que-3-arabinoside, que-3-glucuronide and que-3-rhamnoside content.

Mean quercetin glycoside content of the early leaf removal treatment was 0.345 mg $berry^{-1}$ in 2011 and 0.341 mg $berry^{-1}$ in 2012, however, quercetin glycoside concentration in the berry skins of the control treatment was about 20 % higher in 2011 than in 2012 (Tab. 4 and supplemental Table). Berries shaded before veraison also showed higher concentration of quercetin glycosides in 2011 than in 2012 (0.651 mg g^{-1} berry skin fresh mass and 0.188 mg mg g^{-1} berry skin fresh mass, respectively). This difference may

Table 3

Concentration of amino acids given as mg L^{-1} ± standard deviation at veraison (E-L 34) and harvest, experimental year 2012; Leaf removal: all leaves in the bunch zone removed; Shade: Complete shading by covering bunches with boxes impermeable to light. E-L numbers given after the treatment indicate the developmental stage in which the treatment was applied. Treatment and sampling date effects were evaluated using a generalized linear model (GLM). Different letters indicate significant differences for treatments of all sampling dates according to Fisher's LSD test ($p < 0.05$). Year and sampling date differences are given as asterisks on the right hand side of the table. *) $p < 0.05$; **) $p < 0.01$; ***) $p < 0.001$. "n.d." = not detected; "+" = values are between limit of detection and limit of quantification

Date	10.08.2012					16.10.2012				
Treatment	Leaf removal E-L 27	Control	Shade E-L 27	Leaf removal E-L 27	Leaf removal E-L 34	Control	Shade E-L 34	Shade E-L 27	date sig.
Aspartate									
aspartic acid	41.74 ± 6.08	52.1 ± 2.3	52.69 ± 2.00	7.16 ± 2.3 c	7.13 ± 2.28 c	8.53 ± 1.88 bc	14.34 ± 1.19 a	14.02 ± 3.01 ab	***
asparagine	3.48 ± 0.88	4.51 ± 0.59	7.05 ± 1.99	1.68 ± 1.03 c	1.98 ± 0.18 c	2.6 ± 0.85 c	6.26 ± 0.99 a	4.49 ± 0.96 b	***
methionine	n.d.	n.d.	+	2.52 ± 1.28 c	2.06 ± 0.17 c	3.45 ± 1.65 c	9.25 ± 2.13 a	8.13 ± 1.87 b	***
threonine	7.37 ± 1.35	10.06 ± 0.57	9.35 ± 1.34	21.8 ± 5.82 d	22.36 ± 1.52 cd	30.73 ± 5.07 c	53.64 ± 6.87 a	48.23 ± 5.24 b	***
lysine	+	+	+	+ c	+ c	0.95 ± 0.19 b	1.47 ± 0.03 a	1.2 ± 0.13 b	***
isoleucine	1.13 ± 0.25	1.31 ± 0.03	1.07 ± 0.29	17.08 ± 3.44 b	16.55 ± 1.61 b	20.69 ± 7.13 b	39.34 ± 9.63 a	38.72 ± 4.95 a	***
3-phosphoglycerate									
serine	10.99 ± 2.02	10.98 ± 0.13	7.78 ± 1.23	20.98 ± 2.25 c	23.03 ± 1.26 c	25.76 ± 6.53 bc	39.83 ± 4.98 a	39.42 ± 6.71 ab	***
glycine	+	+	+	1.14 ± 0.1 c	1.25 ± 0.11 c	1.35 ± 0.33 bc	2.11 ± 0.25 a	2 ± 0.17 ab	***
Pyruvate									
alanine	5.17 ± 1.37	8.23 ± 0.54	12.28 ± 3.32	33.12 ± 6.78 c	34.28 ± 2.49 c	39.92 ± 9.25 c	67.28 ± 7.08 a	60.01 ± 12.37 b	***
valine	10.21 ± 2.08	12.08 ± 0.1	11.8 ± 1.67	17.9 ± 4.2 b	17.47 ± 1.44 b	21.22 ± 6.6 b	37.19 ± 8.95 a	35.09 ± 5.31 a	***
leucine	1.73 ± 0.25	2.17 ± 0.21	1.84 ± 0.54	20.72 ± 5.45 b	20.02 ± 2.45 b	26.01 ± 9.92 b	51.8 ± 13.64 a	52.27 ± 7.04 a	***
Shikimate									
tyrosine	3.43 ± 0.47	4.15 ± 0.63	4.53 ± 2.02	2.68 ± 0.38 b	2.88 ± 0.62 b	3.49 ± 0.8 b	6.22 ± 1.46 a	5.6 ± 0.48 a	-
phenylalanine	2.29 ± 0.32	3.24 ± 0.7	2.48 ± 0.51	18.75 ± 4.93 b	17.73 ± 1.52 b	24.26 ± 7.53 b	51.2 ± 11.37 a	46.78 ± 5.49 a	***
tryptophan	10.89 ± 3.93	10.32 ± 0.09	14.5 ± 2.5	5.45 ± 1.2 c	4.5 ± 0.42 c	7.17 ± 2.9 c	19.25 ± 3.95 a	15.62 ± 1.57 b	*
α-Ketoglutarate									
glutamic acid	42.18 ± 3.17	47.41 ± 2.4	50.96 ± 3.04	18.93 ± 5.66 b	23.76 ± 6.32 ab	25.94 ± 5.12 a	28.53 ± 4.01 a	28.00 ± 4.36 a	***
glutamine	30.68 ± 9.66	49.6 ± 1.95	68.82 ± 21.52	33.32 ± 15.36 c	36.57 ± 2.15 c	47.5 ± 15.33 c	132.29 ± 20.23 a	114.96 ± 19.1 b	*
arginine	18.53 ± 7.63	40.13 ± 4.12	34.46 ± 7	28.48 ± 20.94 d	32.27 ± 3.46 d	65.93 ± 8.92 c	164.11 ± 15.39 a	108.66 ± 24.68 b	***
proline	2.51 ± 0.5	3.85 ± 0.19	4.68 ± 0.43	132.55 ± 37.04 b	156.38 ± 26.27 b	190.91 ± 56.85 ab	246.38 ± 57.04 a	260.38 ± 87.73 a	***
histidine	8.91 ± 1.88	10.85 ± 0.64	12.53 ± 2.95	5.82 ± 1.64 b	6.19 ± 0.76 b	8.35 ± 1.81 b	17.4 ± 3.83 a	14.84 ± 1.4 a	-
GABA	6.88 ± 2.66	9.64 ± 1.27	8.99 ± 2.04	83.17 ± 20.84 b	78.61 ± 9.73 b	91.55 ± 16.64 b	139.55 ± 5.12 a	95.96 ± 25.39 b	***
ornithine	+	+	+	+ c	+ c	+ c	1.25 ± 0.17 a	0.74 ± 0.12 b	***
Total amino acids	209.61 ± 41.79	282.36 ± 3.22	307.59 ± 44.17	474.37 ± 135.93 d	506.21 ± 22.78 d	646.85 ± 140.59 c	1128.69 ± 135.68 a	995.1 ± 159.42 b	***
Other									
ammonia	96.82 ± 19.99	112.23 ± 2.26	122.04 ± 18.29	18.35 ± 9.32 d	25.03 ± 1.44 cd	32.69 ± 10.21 bc	53.26 ± 5.56 a	39.17 ± 4.46 ab	***

be attributed to the fact that the shading treatment was applied in a later growth stage in 2011 than in 2012 (E-L 27 in 2012 and E-L 29-31 in 2011) or to higher radiation during the flowering phase (mean PAR during flowering: 258 µmol m^{-2} s^{-1} in 2012 and 428 µmol m^{-2} s^{-1} in 2011).

P C A : A PCA model was constructed with two latent variables explaining 56.29 % of x-block variation. From the scores-plot (Figure, a) it is clearly visible that treatments were effectively separated by PC1 and to a minor extent PC2, while the differences between experimental years were modeled exclusively on the second principal component. The loadings plot (Figure, b) showed that treatments were mainly separated by malic acid and N-OPA, which were more abundant in shaded samples, and quercetin glu-

Table 4

Concentration of phenolics given as µg g^{-1} berry skin fresh weight ± standard deviation at E-L 27, E-L 34 (veraison) and harvest, experimental year 2012. Leaf removal: all leaves in the bunch zone removed; Shade: Complete shading by covering bunches with boxes impermeable to light. E-L numbers given after the treatment indicate the developmental stage in which the treatment was applied. Treatment, year and sampling date effects were evaluated using a generalized linear model (GLM). Different letters indicate significant differences for treatments of all sampling dates according to Fisher's LSD test ($p < 0.05$). Year and sampling date differences are given as asterisks on the right hand side of the table. *) $p < 0.05$; **) $p < 0.01$; ***) $p < 0.001$. "n.d." = not detected; "+" = values are between limit of detection and limit of quantification. GRP = grape reaction product; p-CGT = p-coumaroylglycosyltartrate; Que = quercetin

Date	02.07.2012		10.08.2012				16.10.2012				
Treatment	Control	Leaf removal E-L 27	Control	Shade E-L 27	Leaf removal E-L 27	Leaf removal E-L 34	Control	Shade E-L 34	Shade E-L 27	sign year	sign date
Flavanols											
Procyanidin B1	0.017 ± 0.006	0.054 ± 0.008	0.057 ± 0.03	0.033 ± 0.007	0.05 ± 0.045	0.051 ± 0.016	0.066 ± 0.023	0.057 ± 0.02	0.052 ± 0.025	-	***
Catechin	0.076 ± 0.011	0.119 ± 0.03	0.1 ± 0.015	0.102 ± 0.025	0.097 ± 0.033 a	0.067 ± 0.006 ab	0.074 ± 0.004 ab	0.057 ± 0.004 ab	0.048 ± 0.012 b	-	***
Procyanidin B2	0.048 ± 0.007	0.074 ± 0.021	0.061 ± 0.012	0.056 ± 0.02	0.037 ± 0.006	0.037 ± 0.004	0.034 ± 0.008	0.027 ± 0.005	0.03 ± 0.016	-	***
Epicatechin	0.013 ± 0.004	0.025 ± 0.008	0.034 ± 0.007	0.024 ± 0.007	0.017 ± 0.008	0.009 ± 0	0.015 ± 0.002	0.01 ± 0.002	0.013 ± 0.006	-	***
Total Flavanols	0.154 ± 0.013	0.271 ± 0.064	0.251 ± 0.013	0.215 ± 0.058	0.2 ± 0.051	0.164 ± 0.015	0.188 ± 0.023	0.152 ± 0.012	0.143 ± 0.054	-	***
Hydroxycinnamic acids											
Coumaroylglucose	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	***	-
Caftaric Acid	0.53 ± 0.057	0.597 ± 0.051	0.577 ± 0.064	0.583 ± 0.042	0.519 ± 0.064 a	0.386 ± 0.043 b	0.495 ± 0.039 b	0.477 ± 0.027 b	0.508 ± 0.094 b	-	*
GRP	0.003 ± 0	0.002 ± 0	n.d.	0.001 ± 0.001	0.003 ± 0.002 b	0.004 ± 0.001 a	0.001 ± 0 b	n.d. b	n.d. b	*	-
p-CGT	0.009 ± 0.003	0.014 ± 0.002	0.013 ± 0.003	0.006 ± 0.001	0.009 ± 0.008 b	0.017 ± 0.001 a	0.008 ± 0.001 b	0.007 ± 0.001 c	0.008 ± 0.006 bc	-	-
Coutaric acid	0.322 ± 0.036	0.382 ± 0.036	0.351 ± 0.056	0.307 ± 0.025	0.256 ± 0.037 b	0.177 ± 0.041 b	0.229 ± 0.021 b	0.229 ± 0.016 a	0.182 ± 0.042 b	-	***
Fertaric acid	0.016 ± 0.002	0.026 ± 0.002	0.033 ± 0.003	0.039 ± 0.001	0.038 ± 0.005 b	0.048 ± 0.005 a	0.03 ± 0.002 cd	0.024 ± 0.004 d	0.035 ± 0.003 c	***	***
Caffeic acid	0.004 ± 0.002	0.006 ± 0.001	0.004 ± 0.001	0.005 ± 0.002	0.004 ± 0.003 a	0.007 ± 0.001 a	0.006 ± 0.002 a	0.004 ± 0.001 a	0.003 ± 0.001 b	*	-
Cumaric acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+	+	***	-
Total Hydroxycin. acids	0.883 ± 0.096	1.027 ± 0.088	0.979 ± 0.124	0.941 ± 0.069	0.83 ± 0.097	0.64 ± 0.079	0.769 ± 0.057	0.742 ± 0.043	0.736 ± 0.14	***	-
Flavonols											
Que-3-rutinoside	0.058 ± 0.006	0.176 ± 0.052	0.026 ± 0.018	0.034 ± 0.013	0.271 ± 0.023 a	0.134 ± 0.048 b	0.083 ± 0.01 b	0.072 ± 0.023 c	0.015 ± 0.004 c	***	*
Que-3-galactoside	0.015 ± 0.002	0.037 ± 0.011	0.005 ± 0.004	0.009 ± 0.003	0.237 ± 0.032 b	0.286 ± 0.058 a	0.127 ± 0.016 c	0.016 ± 0.006 d	0.006 ± 0.001 e	-	***
Que-3-glucoside	0.033 ± 0.004	0.067 ± 0.018	0.011 ± 0.007	0.02 ± 0.006	0.783 ± 0.055 b	0.906 ± 0.112 a	0.476 ± 0.056 c	0.039 ± 0.012 d	0.032 ± 0.005 e	-	***
Que-3-glucuronide	0.384 ± 0.018	0.61 ± 0.113	0.188 ± 0.102	0.227 ± 0.034	0.884 ± 0.084 a	0.492 ± 0.132 b	0.375 ± 0.01 bc	0.343 ± 0.037 c	0.127 ± 0.025 c	**	*
Que-3-xyloside	0.003 ± 0	0.005 ± 0.001	0.002 ± 0.001	0.003 ± 0.001	0.012 ± 0.02 b	0.03 ± 0.004 a	0.014 ± 0.001 bc	0.004 ± 0.001 cd	0.003 ± 0.001 d	-	*
Que-3-arabinoside	0.01 ± 0.002	0.036 ± 0.011	0.003 ± 0.003	0.004 ± 0.002	0.262 ± 0.013 a	0.259 ± 0.07 a	0.099 ± 0.013 b	0.013 ± 0.007 c	0.002 ± 0.001 d	-	***
Que-3-rhamnoside	0.005 ± 0.001	0.014 ± 0.003	0.002 ± 0.002	0.004 ± 0.001	0.652 ± 0.056 b	0.765 ± 0.176 a	0.312 ± 0.052 c	0.007 ± 0.003 d	0.002 ± 0.001 d	-	***
Total Flavonols	0.508 ± 0.015	0.945 ± 0.208	0.237 ± 0.138	0.301 ± 0.058	3.101 ± 0.093 a	2.871 ± 0.551 b	1.485 ± 0.141 c	0.495 ± 0.086 d	0.188 ± 0.033 d	-	***
Total Phenolics	1.544 ± 0.115	2.243 ± 0.339	1.467 ± 0.248	1.457 ± 0.068	4.131 ± 0.131 a	3.675 ± 0.54 a	2.442 ± 0.073 b	1.388 ± 0.12 c	1.068 ± 0.166 c	*	***

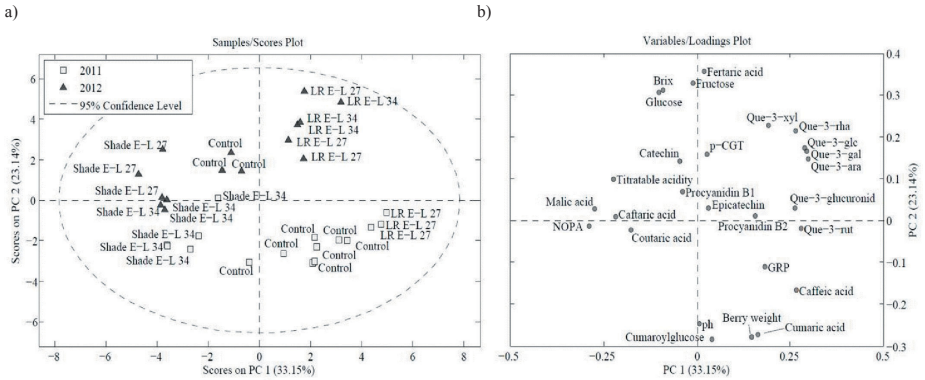

Figure: Scores and loadings plots of the principal component analysis (PCA) conducted on 2011 and 2012 measurements at harvest. LR: all leaves in the bunch zone removed; Shade: Complete shading by covering bunches with boxes impermeable to light. E-L numbers given after the treatment indicate the developmental stage in which the treatment was applied. GRP = grape reaction product ; p-CGT = p-coumaroylglycosyltartrate ; Que = quercetin.

cosides, which were more concentrated in leaf removal samples. Flavanols had the smallest influence on sample separation, while hydroxycinnamic acids, except coutaric and caftaric acid, showed strong loadings on PC2, which separates years, but not treatments. Malic acid and N-OPA were strongly positively correlated. Both correlated negatively with quercetin-glycosides, mainly quercetin-3-glucuronide and quercetin-3-runtinoside. Berry weight correlated positively with coumaric acid and negatively with sugars. Univariate correlation analysis confirmed these results.

Discussion

Several studies have investigated the effects of microclimate manipulation on berry quality traits, mainly focusing on the effects of the qualitatively important phenolics in red winegrape production. Some of these studies have found an effect of microclimate manipulation on grape ripeness (°Brix), while others did not find a significant effect. The results obtained in our study are in accordance with some studies published on post-flowering leaf removal (e.g. (Molitor *et al.* 2011)) or using an artificial shading methodology after anthesis (Spayd *et al.* 2002, Downey *et al.* 2004), while standing in contrast to others (Dokoozlian and Kliewer 1996, Koyama *et al.* 2012). In the latter studies, significant changes in berry weight and sugar concentration have been observed after artificially shading berries at the beginning of flowering and directly after berry set, respectively. In our study, however, the shading treatment was only applied about 14 d after flowering. Therefore, the treatments in our study might have been applied at a developmental stage in which berry size had been determined already. Although temperatures in the boxes and ambient temperatures were similar (Downey *et al.* 2004, Koyama *et al.* 2012), it has to be stressed that berry temperatures in the boxes are different to the temperature of exposed berries, as the shaded berries are not heated up by solar radiation. Berries grown in boxes are therefore exposed to a compressed diurnal temperature range and diminished light and temperature stress, which may hasten berry development (Spayd *et al.* 2002, Cohen *et al.* 2012) and therefore compensate growth deficits induced by bunch shading.

Similar to the shaded bunches, no differences were found in sugar accumulation of bunches from leaf removal vines. Vines can compensate the reduction in leaf area caused by leaf removal by mobilization of reserve carbohydrates, an increase in photosynthetic activity and stronger growth of lateral shoots (Poni *et al.* 2006). The leaf removal intensity applied in our study might not have been severe enough to overcome these compensatory effects and influence berry size and sugar content of the leaf removal treatment significantly.

Elevated malic acid concentrations were detected in shading treatments in both years. Malic acid is respired at a higher rate at high berry temperatures (Lakso and Kliewer 1975), which explains the differences found between shaded and control or exposed berries. Only in samples shaded at veraison the differences in malic acid led to a significantly elevated level of titratable acidity. In contrast to malic acid concentration, pH values appeared to decline with increasing sun exposure, a fact that has previously been reported for Spanish vineyards (Martinez de Toda and Balda 2014) and may be related to decreased potassium concentrations in exposed berries, as reported by Smart *et al.* (1985).

In general, shaded samples showed a higher concentration of amino acids and total nitrogen than control or defoliated samples, which is in accordance with other studies (Schultz *et al.* 1998, Kliewer and Ough 1970). Although berry skins and juice have been analyzed in this study, the grape seeds, as one of the largest nitrogen depots of the berry (about 500 µg N berry^{-1}, calculated using seed

N concentrations from Castrotta and Canella (1978) and Fantozzi (1981)), were not analyzed. More research will therefore be necessary to clarify if equal amounts of N-containing compounds are transferred to the berries and the N-compounds undergo a different fate, e.g. accelerated transport to the seeds, or if the N transport into the berry is modified by grape microclimate. Although some microclimatic effects on single amino acids could be shown before veraison, the changes induced by microclimatic differences were more pronounced after veraison, when significant differences were measured for all amino acids except glutamic acid and proline. The standard deviations for field replicates of amino acids were rather large when compared to the ones obtained for berry phenolics, indicating that factors other than light play a stronger role in amino acid than in phenolic accumulation.

On average, amino acid concentration of control samples and shaded samples was 30 % and 120 %, respectively, elevated as compared to fully exposed samples. Differences between the timing of treatment application were only marginal. Thus, the post-veraison period seems to be crucial for light influence on amino acid synthesis. Both amino acid and ammonia concentration in fully exposed samples of our experiment can be regarded as insufficient for yeast nutrition (Ribereau-Gayon *et al*. 2006).

A clear temporal pattern was observed in the accumulation of the various classes of phenolics. Flavanols and most hydroxycinnamic acids accumulated mainly before veraison, while the main querctin glycoside accumulation occurred post-veraison. The synthesis of quercetin glycosides seemed to follow the interception of direct radiation of the grapes in an almost linear way. At harvest, total phenolic content of all treatments differed significantly, with the exception that there was no significant difference between the two shading treatments. From these results it can be concluded that the timing of leaf removal treatments does influence the content of phenolics of the grapes at harvest. This effect can almost exclusively (to about 95 % on average) be explained by the accumulation of quercetin glycosides induced by excess light, which has been observed pre- and post veraison.

Shading and leaf removal did not influence the level of flavanol accumulation, except for catechin, the content of which was moderately increased by light interception at harvest in 2012. Other authors have shown light-induced effects on flavanol accumulation when treatments were applied directly at the beginning of flowering (Koyama *et al.* 2012). Therefore, it appears likely that the enzymatic setup for flavonol synthesis takes place during flowering and shortly afterwards, and can be influenced by light only then. Flavanol content of the berries then continues to increase, but is no longer subject to light influence. The concentration of flavanols was similar in both experimental years. Compared to flavonol accumulation, light influence on flavanol accumulation is relatively weak in red grapes (Koyama *et al.* 2012), which is in accordance with our results. Similar to flavanol accumulation, little light influence was measured on the accumulation of hydroxycinnamic acids. Although it has been shown that hydroxycinnamic acid synthesis is influenced by light in other species, like *Echinacea purpurea* (Abbasi *et al*. 2007) little such data are available for *Vitis vinifera*. The content of hydroxycinnamic acids was decreased by shading before veraison in 2012. However, the effects of light exposure and shading remained inconsistent during the experimental years, as no light influence was detected in 2011. Just as flavanol synthesis, hydroxycinnamic acid synthesis occurs mainly pre-veraison, and an earlier onset of the experiment may have revealed light influence on the synthesis of these compounds at earlier developmental stages.

Flavonol accumulation in control and leaf removal samples occurred during the entire experimental period, the main phase of accumulation being post-veraison. While other authors (Downey *et al.* 2004, Koyama *et al.* 2012) observed a decreasing content and concentration of flavonols in shaded bunches of 'Shiraz' and 'Cabernet Sauvignon', the quercetin glycoside content of the berries in our study was not decreased by shading, but remained remarkably stable. Shading virtually "froze" the content of the respective glycosides, giving an exact picture of the flavonol profile at the time the shading was applied. For example, almost no quercetin glycosides except que-3-glucuronide and que-3-rutinoside were present in the early shading treatments of both experimental years as well as in the berries sampled at the beginning of the experiment and at veraison in 2012. While que-3-glucuronide and que-3-rutinoside were already present at the beginning of the experiment, leaf removal or shading after veraison did not significantly change the levels of these flavonols at harvest. On the other hand, our data suggest that the accumulation of que-3-rhamnoside occurs almost exclusively after veraison, and was little influenced even by leaf removal before veraison. A similar pattern was also shown for que-3-arabinoside and que-3-galactoside. Hence, it is highly likely that the accumulation of specific flavonol glycosides underlies strong developmental regulation, in accordance with data published by Ono *et al.* (2010), who show the developmental regulation of two flavonol glycosyltransferases. Nevertheless, the function of the various quercetin glycosides in the berry is yet to be clarified and deserves further research.

The strong negative correlation between the accumulation of phenolics and amino acids underlines the tight relation of both metabolic pathways. However, as in this study the light-induced flavonols are the main contributor to the phenolic profile of 'Riesling' and juice amino acids are decreased by radiation, the strong correlation between light, phenolics and amino acids is not surprising. It has been shown that reactions to oxidative stress and nitrogen deficiency are similar (Keller and Hrazdina 1998, Lea *et al.* 2007), and share, at least partially, a common signaling pathway (Harding *et al.* 2003). Further, nitrate inhibits the synthesis of phenolics in grape tissue cultures (Pirie and Mullins 1976). At least at veraison, NH_4-Nitrogen contribution to the nitrogen pool was elevated in berries exposed to high levels of radiation by leaf removal. This may be a hint that there but metabolic pathways may compete for carbon skeletons, which are limiting for ammonia

integration into the amino acid metabolism. Nevertheless, more research is needed to clarify whether there is indeed a common control of both pathways, or if the regulation of both pathways occurs independently of each other.

Conclusion

Compositional changes in white 'Riesling' induced by leaf removal were observed for the flavonoids, amino acids and malic acid. These changes can be attributed to the effect of increased light interception by the grapes. The changes in leaf-fruit ratio showed no significant effects on sugar accumulation, nor did shading of the bunches. Early (E-L 27) leaf removal was shown to increase the skin content of quercetin glycosides and some hydroxycinnamic acids already before veraison. The differences in skin quercetin glycoside content between early and late leaf removal were still measurable at harvest. Early leaf removal of 'Riesling' grapes may therefore increase the bitter perception in the resulting wine, especially when there are long skin contact times during processing. By excluding the influence of light from an early developmental stadium, the synthesis of quercetin glycosides was inhibited completely. Manipulation of the grape microclimate affected the concentration of some amino acids already at veraison, however much stronger effects were observed post-veraison. Leaf removal before or at veraison may lead to low yeast available nitrogen and therefore increase the risk of stuck or sluggish fermentations.

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Chapter 4

Light promotes expression of monoterpene and flavonol metabolic genes and enhances flavour of winegrape berries (*Vitis vinifera* L. cv. Riesling)[3]

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Light promotes expression of monoterpene and flavonol metabolic genes and enhances flavour of winegrape berries (*Vitis vinifera* L. cv. Riesling)

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Abstract

Background and Aims: Insolation of grape bunches has long been postulated to enhance the flavour of aromatic grape cultivars. This hypothesis was tested by combining gene expression and metabolic analysis of the monoterpene and flavonol synthesis pathways.

Methods and Results: Grape bunches were shaded or shaded and reilluminated to investigate the influence of light on the monoterpene and flavonol biosynthetic pathways. The expression of terpenoid and flavonol metabolic genes was measured by quantitative polymerase chain reaction under light and shade conditions during the ripening phase and compared to the accumulation of their respective metabolic products. Expression of flavonol synthase and flavonol glycosyltransferase genes was virtually absent in shaded bunches, but expression increased strongly upon reillumination, as did the flavonol content of the berries. The expression of the terpene synthase genes as well as the monoterpene content were greatly reduced in shaded bunches, and then increased upon reillumination. The expression of terpene glycosyltransferases was affected only slightly by light.

Conclusions: The results of this study show the positive influence of light on monoterpene and flavonol biosynthesis.

Significance of the Study: Optimising the light exposure of grape berries enables the viticulturist to manipulate grape berry aroma and consequently the composition of wine.

Keywords*: aroma, flavonol, flavonol synthase, glycosyltransferase, light influence, monoterpene, monoterpene synthase, shading*

Introduction

Terpenoids and phenolic substances are classes of secondary metabolites that strongly influence the sensory properties of grape berries. Terpenoids are structurally derived from isoprene units. Most of these metabolites are found in the plant kingdom (Kouloura et al. 2014). They are considered to play a key role in plant communication and are associated with general stress and defence reactions (Gershenzon and Dudareva 2007). Being widely regarded as positive contributors to the flavour of a wide variety of fruits and flowers, there is a strong commercial and scientific interest in factors influencing terpenoid accumulation in plants (Schwab et al. 2008). The monoterpenes of grapes and wine are long known to contribute substantially to their varietal typicity and sensory properties (Rapp and Mandery 1986, Guth 1997). All terpenoids are synthesised from isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Isopentenyl diphosphate and DMAPP can be synthesised via two independent pathways: the cytosolic mevalonate and the plastidic mevalonate-independent methylerythritol phosphate (MEP) pathway. The majority of monoterpenes (C10), which play a crucial role in the aroma of grape berries, are synthesised from IPP and DMAPP derived from the MEP pathway (Bohlmann and Keeling 2008). The direct precursors of terpenoids, geranyl diphosphate (C10, geranyl pyrophosphate), farnesyl diphosphate (C15, farnesyl pyrophosphate) and geranylgeranyl diphosphate (C20, geranylgeranyl pyrophosphate), are synthesised from IPP and DMAPP. Terpene synthases catalyse the formation of monoterpenes from their respective direct percursors (Figure 1). The final monoterpene pattern found in plants arises from further modification of many of these terpenes by oxidation, glycosylation, acylation and other reactions.

Recent analysis of the grapevine genome showed the presence of a large family of terpene synthases in *Vitis vinifera* L. cv. Pinot Noir (Martin et al. 2010). While expression of the majority of *VvTPS* genes, and accumulation of the corresponding terpenoids, peaks around flowering, the *VvTPS-g* subfamily shows a second peak of gene expression around the developmental stage of berry softening (Martin et al. 2012, Matarese et al. 2013). This *VvTPS* subfamily encodes enzymes responsible for the formation of the monoterpenols, linalool, nerol and geraniol, which impact the flavour of aromatic and semi-aromatic grape cultivars, such as Gewürztaminer, Riesling, Müller-Thurgau and several muscat cultivars. It appears that the expression of most terpene synthase genes declines during ripening (Martin et al. 2012, Matarese et al. 2013). Monoterpenes are, however, accumulated throughout the ripening phase, reaching their peak concentration at maturity (Wilson et al. 1984, Park et al. 1991).

The majority of terpenoids in grapes are present in their glycosylated form (Günata et al. 1985, Park et al. 1991, Belancic et al. 1997), linked either to glucose or to glucose and a second sugar moiety (Williams et al. 1982, Mateo and Jiménez

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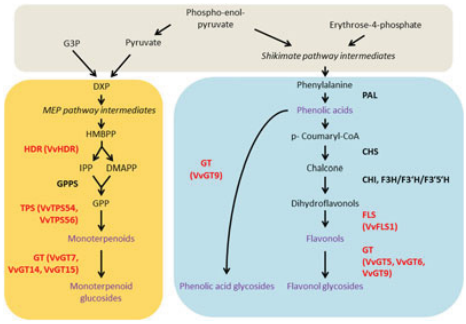

Figure 1. Schematic illustration of the monoterpene and phenylpropanoid metabolism. Enzymes of which the corresponding gene expression has been analysed and analysed metabolites are marked in red and purple, respectively. Orange box. Methylerythritol phosphate (MEP) pathway and synthesis of monoterpenes: DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; GPP, geranyl pyrophosphate; GPPS, geranyl pyrophosphate synthase; GT, glycosyltransferase; HMBPP, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate; HDR, HMBPP reductase; IPP, isopentenyl diphosphate; TPS, terpene synthase. Blue box. Synthesis of phenylpropanoid and flavonoids: CHI, chalcone isomerase; CHS, chalcone synthase; F3H/F3'H/F3'5'H, flavanone-3-hydroxylases; FLS, flavonolsynthase; GT, glycosyltransferase; PAL, phenylalanine ammonia lyase. Grey box. Primary metabolism: G3P, glyceraldehyde 3-phosphate.

2000). Glycosylation greatly increases the water solubility of terpenes, making the storage of large amounts of terpenoids possible. Glycosylated terpenoids are odourless but can be hydrolysed to their odour-active form by enzymatic or acid hydrolysis. This occurs during winemaking and wine ageing, when terpenoid glycosides are hydrolysed by yeast and bacterial glycosidases or, much slower, by acid hydrolysis at the low pH of wine and grape juice (Moreno-Arribas and Polo 2009). Glycosylated terpenoids are therefore regarded as the hidden aromatic potential of wine. Only recently, the first monoterpene glycosyltransferases (GTs) from *V. vinifera* have been functionally characterised in vitro (VvGT7, VvGT14 and VvGT15) (Bönisch et al. 2014a, b). Activity-based metabolic profiling revealed a high sugar acceptor promiscuity of terpenoid GTs in *V. vinifera* (Bönisch et al. 2014a).

Phenolic substances or phenylpropanoids represent one of the most abundant and diverse classes of secondary metabolites in the plant kingdom, with more than 8000 structures currently known (Dai and Mumper 2010, Tsao 2010). Grape phenolic substances, including non-flavonoid hydroxycinnamic acids and flavonoids, are synthesised via the shikimate pathway. While esterification, hydroxylation, methylation or glycosylation of cinnamic acid gives rise to the class of hydroxycinnamic acids, flavonoids are synthesised from coumaryl-CoA and three malonyl-CoA units. Important classes of flavonoids found in grapevine tissues are flavanols (flavan-3-ols), flavonols and anthocyanins, the latter being absent in berries of white grape cultivars. Methylation, esterification, hydroxylation, glycosylation or condensation gives rise to a vast array of flavonoids of the respective classes in grape berries. Plant phenolic substances are generally associated with defence against pathogens and herbivores, pigmentation and protection against excessive irradiation in various parts of the electromagnetic spectrum. In the latter action, flavonols are of particular importance as they protect the plant from UV radiation (Kolb et al. 2003). Flavonol biosynthesis from dihydroflavonols is catalysed by flavonol synthases (FLSs). The velocity with which *FLS* gene expression and flavonol accumulation are up-regulated by illumination underlines their importance in UV protection (Matus et al. 2009). Glycosyltransferases, utilising hydroxycinnamic acids, flavanols and flavonols as sugar acceptors, have been functionally characterised (VvGT1, 5, 6, 9, 10 and 11) (Ford et al. 1998, Jánváry et al. 2009, Ono et al. 2010, Khater et al. 2011, Bönisch et al. 2014b). It has been shown that in an *Arabidopsis thaliana* mutant compromised in flavonol-3-O-glycosylation, the entire flavonol metabolism was down-regulated by feedback inhibition (Yin et al. 2012). A schematic overview of both terpenoid and phenylpropanoid metabolism is given in Figure 1.

While contributing positively to colour, shelf-life and taste of red wine, phenolic substances are often considered as negative contributors to white wine sensory attributes, leading to increased bitterness, astringency (Singleton et al. 1975, Arnold et al. 1980) and browning (Singleton 1987). Further, phenolic substances in wine are known to form complexes with proteins, leading to haze formation (Ferreira et al. 2001). Phenolic substances, however, may also contribute to ageing stability and increase the shelf-life of white wines. Apart from their technical and sensory properties, the important health benefits displayed by plant phenolic substances have sparked great scientific and technological interest in these secondary metabolites (Yao et al. 2004).

Insolation has been shown to affect almost every aspect of berry composition. Various studies have shown the effect of light on berry growth (Dokoozlian and Kliewer 1996) and on the concentration and profile of anthocyanins (Downey et al. 2004), flavonols (Price et al. 1995, Bergqvist et al. 2001), minerals and amino acids (Pereira et al. 2006), norisoprenoids (Marais et al. 1992, Lee et al. 2007) and terpenoids (Reynolds and Wardle 1989, Skinkis et al. 2010). Research on the effect of insolation on the accumulation of monoterpenes has, however, been sparse and sometimes limited in its analytical approach. Further, in many studies dealing with terpene concentration under different light conditions, the concentration of sugars varies significantly between treatments. The accumulation of terpenoids is strongly correlated with the progress of ripening; accordingly, a significant difference in the concentration of sugars can mark a major source of bias in these experiments, and therefore, their results have been doubted (Luan et al. 2006).

No study to date has combined metabolomic and gene expression analysis under different microclimatic conditions to study their influence on terpenoid metabolism in grapes. In this study, we investigate the influence of light on the expression of terpene synthases and terpene GTs in Riesling grapes throughout grape ripening and compare the gene expression patterns to the accumulation of monoterpenes in the berries. In addition, transcript levels of flavonol biosynthetic genes and the accumulation of phenolic substances were measured, as recent data (Kang et al. 2014) suggest a possible metabolic crosstalk of these two important aspects of grape composition. Gaining a better understanding of the synthesis of secondary metabolites will lead to sound recommendations to the industry on growing fruit to a specification desired for distinct wine styles.

Materials and methods

Experimental site

Field experiments were undertaken in the 2012 growing season using non-irrigated Riesling [clone 198–25Gm; grafted on rootstock SO4 Gm47 (*Vitis berlandieri x V. riparia*)] in a

research vineyard located close to Geisenheim, Germany (49° 59′20″N; 7°55′56″E). The region has a cool climate compared to that of other viticultural regions, with an avarage temperature during the growing season of 15.2°C and average rainfall of 550 mm/year. The experimental year was an average year with a mean temperature of 15.2°C during the growing season and a rather dry and hot ripening period, leading to moderate water stress during the early ripening phase. Vines were trained to a vertical shoot position (VSP)-type canopy system in a north–south row orientation (row azimuth 164°). Row spacing was 2.10 m and vine spacing 1.05 m. In order to obtain a homogeneous canopy, the shoot number was adjusted to ten shoots per vine. Average yield in the experimental year was about 3.5 kg/vine.

Field trial

A field trial was established in a randomised block design with four replicates. Each replicate consisted of four vines. Three treatments were established: (i) one control treatment grown under regular field conditions; (ii) one shading treatment in which bunches were sheltered in lightproof boxes (Downey et al. 2004) at veraison [60% of berries softened, 17 August 2012, 66 days after full flowering (DAF)]; and (iii) a shading/light treatment, in which bunches were sheltered in boxes until 34 days after veraison (20 September 2012, 100 DAF) and then exposed to light until harvest by removing the boxes in the morning of the 20 September 2012. This treatment was sampled after 8 h and 20 days of light exposure. To ensure that bunches sampled in the experiment were influenced by a similar light environment, only bunches exposed to the western side of the canopy at a height of 80–110 cm above-ground were either packed in boxes or used as control bunches. As bunches sheltered in boxes cannot be reached by botryticide spraying, the boxes were opened at night and sprayed on the day on which the regular botryticide spraying was applied. Every vine and bunch were sampled only once, to avoid wounding reactions due to multiple sampling of the same bunch. The vines were sampled 86 DAF on 6 September 2012, 100 DAF on 20 September 2012 and 119 DAF at harvest on 10 October 2012.

Temperature and humidity were monitored by placing three probes (Lascar Electronics, Salisbury, England) inside the boxes and in the surrounding canopy for the duration of the experiment. Incident radiation in the boxes was measured by inserting three LI-190 SA50 Quantum Sensors (Li-Cor, Lincoln, NB, USA) connected to a LI-1400 data logger inside the boxes. These data were compared with ambient photon flux density. Sample size was 120 berries per replicate – 30 berries from each of four bunches (one bunch per vine) were randomly sampled. All sampling was conducted at the same time each night, and berries were frozen in liquid nitrogen immediately after sampling. Samples were stored at −80°C until analysis.

Experimental conditions

Temperature and humidity measured in the boxes were similar to values obtained by measurements in the canopy (Figure S1), which agrees with results obtained by Downey et al. (2004) and Koyama et al. (2012). As for light exclusion, only about 1% of ambient photon flux density was measured in the boxes (Figure S1). These measurements are almost identical with the values reported by Downey et al. (2004). Point quadrat analysis (Smart and Robinson 1991) showed that canopy conditions (number of leaf layers and number of shaded leaves) in the four blocks were not significantly different. The average number of leaf layers was two, with homogeneous distribution along the trellis. Vines showed medium vigour, probably related to a period of moderate water stress during the ripening phase. About 45% of bunches were exposed to direct sunlight.

Solar radiation absorbed in the bunch zone was assessed using a radiation distribution model based on tracing of a random sample of photons in conjunction with the Monte Carlo method. The model was based on the grapevine radiation model published by Hofmann et al. (2014) and adapted to calculate only the radiant energy density absorbed by the area of the bunch zone in which sampling was conducted (80–110 cm above-ground, western side of the canopy). For the calculations, diffuse and global radiation data supplied by the German weather service (DWD) station in Geisenheim, located approximately 2 km from the experimental vineyard, were used. Mean and total absorbed radiant energy for the treatments are shown in Table 1.

Sample preparation

The frozen berries were pre-ground with a household blender. About 8 g of the blended sample was fine-ground under liquid nitrogen with a mixer mill (MM 400; Retsch, Haan,Germany); 0.1 g of the fine-ground sample was used for RNA extraction and 2 g for the analysis of phenolic substances. The rest of the pre-ground sample was used for refractrometric, Fourier transform infrared spectroscopy and GC–MS analyses.

Using the Gene Matrix universal RNA purification kit (EURx, Gdansk, Poland), RNA was extracted from grape berries that were ground to fine powder following the protocol for plant tissue RNA purification. Final RNA concentration was determined by UV–VIS spectrometry in a NanoDrop spectrophotometer (Thermo Fisher, Dreieich, Germany).

Table 1. Effect of shading and light treatments on the mean and total radiant energy absorbed by the bunch zone on the western side of the canopy as estimated by a Monte Carlo simulation.

Time span	Radiant energy (mJ/m^3)					
	Control		Shade		Shade/light	
	Mean [$mJ/(day \cdot m^3)$]	Total (mJ/m^3)	Mean [$mJ/(day \cdot m^3)$]	Total (mJ/m^3)	Mean [$mJ/(day \cdot m^3)$]	Total (mJ/m^3)
17.08–06.09.12	9.22	193.60	0.18	3.87	0.18	3.87
06.09–20.09.12	7.99	305.42	0.16	6.11	0.16/8.27†	14.38
20.09–09.10.12	5.26	405.41	0.11	8.11	5.26	122.80

†Data displayed are the mean radiant energy absorption before and after exposure to light. Control, bunches permanently exposed to light; Mean, mean radiation per day; Shade, sunlight permanently excluded from bunches by sheltering in lightproof boxes; Shade/light, bunches shaded until the morning of 20 September 2012, then exposed to light by removing boxes; Total, total radiation intercepted from the beginning of the experiment.

Transcription analysis of GTs

The gene expression patterns of 14 *GT* genes (*VvGTs 7–20*) were analysed together with five reference genes (*VviActin*, *VviAP47*, *VviPP2A*, *VviSAND* and *VviTIP41*) using the Genome Lab GeXP Genetic Analysis System (Beckman Coulter, Krefeld, Germany), a multiplex, quantitative gene expression analysis system. A detailed description of the method employed has been published by Boenisch et al. (2014b) and was not modified. Primer sequences and the concentration of the primers are available in Tables S1 and S2.

Raw data were analysed using the Fragment Analysis tool. The fragment data of the standard curves and samples were then normalised to the peak area of KAN^r RNA with the Express Analysis tool. Subsequently, the relative signal level of each sample replicate was interpolated from the standard curve. The data were further normalised to the geometric mean of the five reference genes with the Quant tool. All software for GeXP data analysis was purchased from Beckman Coulter.

Transcription analysis of TPS and GTs by quantitative real-time PCR

The genes *VvTPS54*, *VvTPS56*, *VvHDR*, *VvFLS1*, *VvGT5*, *VvGT6*, *VvGT7* and *VvGT9* were further analysed by quantitative real-time PCR. The cDNA of *VvActin* was also quantified as the reference gene. The cDNA was synthesised from 225 ng of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) in a total volume of 20 µL according to the manufacturer's instructions. Real-time PCR was carried out using iQ SYBR Green Supermix (Bio-Rad Laboratories) following the manufacturer's instructions with one-eightieth of the reaction consisting of cDNA and a final primer concentration of 250 nmol/L in a final volume of 25 µL. With *VvGT5* and *VvGT6*, the method was modified, and primer concentration was halved and cDNA concentration doubled. Two technical replicates of each sample were run on the iQ5 RT-PCR Detection System (Bio-Rad Laboratories). The efficiencies of the PCR were calculated from a serial dilution series of a pool of all cDNAs with the iQ5 software. Relative normalised quantities were calculated from quantification cycle values applying a modified delta-delta-Ct method using an in-house routine. Primer pairs except for *VvActin* (Reid et al. 2006), *VvGT5*, *VvGT6* (Ono et al. 2010), *VvTPS54*, *VvTPS56*, *VvHDR* (Martin et al. 2012) and *VvFLS1* (Downey et al. 2003) were designed using the tool Primer-BLAST (Ye et al. 2012). All *VvGT* and *VvActin* primers were purchased from Eurofins MWG Operon (Ebersberg, Germany). All other primers were purchased from biomers.net (Ulm, Germany). Primer sequences are given in Table S1.

Analysis of phenolic substances by HPLC

For the analysis of berry phenolic substances, 2 g of the fine-ground berries was defrosted and centrifuged at 12 000× *g* for 5 min. The supernatant was carefully transferred to a new vessel and centrifuged again for 5 min at 12 000× *g*. This step was repeated four times. The remaining liquid was filtered through a 45 µm syringe filter and submitted directly to HPLC analysis. The extracts were analysed by an Accela HPLC/diode array detection system coupled to an LXQ MS (Thermo Fisher). Chromatographic separation was achieved on a 150 × 2 mm i.d., 3-µm Luna 3u C18 100-Å column (Phenomenex, Torrance, CA, USA) protected with a guard column of the same material. Injection volume was 3 µL, at a flow rate of 250 µL/min. Elution conditions were solvent A 2% acetic acid and solvent B acetonitrile/water/acetic acid (50:50:0.5 v/v/v). Gradient elution was applied: 0–20 min from 96–50% solvent A and 4–50% solvent B and 20–23.1 min to 100% B, washing with 100% B for 2 min before reequilibrating the column. The following MS conditions were applied: electrospray ionisation (ESI) source voltage −3.00 kV during negative and +5.00 kV during positive ionisation mode, capillary temperature 275° C and collision energy for MS^n-experiments 35% (arbitrary units). Detection wavelength was 280 nm for flavanols, 320 nm for phenolcarbonic acids and 360 nm for flavonols. Peak identification was based on a combination of HPLC retention time and UV spectra as well as MS data. Compounds were quantified by using peak area from external calibration curves. All standard sources are presented in Table S3. Where no standards were available, substances were quantified using the calibration for the closest phenolic relative (caftaric acid as caffeic acid; fertaric acid as ferulic acid, coutaric acid and *p*-coumaroyl-glycosyl-tartrate as coumaric acid).

Analysis of monoterpenes by GC–MS

Crushed berries (100 g) were thawed overnight at 4°C, mixed with a household blender and centrifuged for 10 min at 10°C and 4600× *g*. The supernatant was carefully decanted from the centrifugation vessel, and 40 mL of the juice was clarified with 5 mL Carrez I [150 g potassium hexacyanoferrate (II) trihydrate/L] and 5 mL Carrez II (300 g zinc sulfate heptahydrate/L) solution, filled up to 100 mL and filtered. Monoterpenes were analysed by solid-phase extraction-GC/MS, following a method similar to that of Di Stefano (1991). 2-Octanol was used as internal standard (30 mg 2-octanol/100 mL CH_2Cl_2). Solid-phase extraction cartridges (Strata-X; Phenomenex) were conditioned with 10 mL CH_2Cl_2, followed by 5 mL MeOH and equilibrated with 5 mL H_2O. Clarified juice (30 mL) was added onto the prepared cartridges. Cartridges were dried under air flow for 30 min. Free monoterpenes were eluted with 10 mL CH_2Cl_2. Internal standard (25 µL) was added and residual water removed by adding anhydrous sodium sulfate. Extracts were concentrated in a vacuum evaporator (Büchi, Flawil, Switzerland) at 30°C and 520 hPa. The concentrate was transferred to a 200 µL GC vial inlet for analysis. Glycosylated monoterpenes were eluted with 5 mL MeOH and desiccated in a vacuum evaporator at 45°C and 100 hPa. The dried methanol eluent was resuspended with citrate buffer (5 mL, pH 4.0), and 25 mg β-glycosidase (VP 1050-1; Erbslöh, Geisenheim, Germany) was added. Reaction time was 12 h at room temperature under nitrogen atmosphere in the dark. After the glycosidase reaction internal standard (25 µL) and 2 mL methyl *tert*-butyl ether were added, and the mixture was vortexed for 30 s and subsequently centrifuged (room temperature, 1600× *g*, 10 min). The supernatant was transferred to a 200 µL GC vial inlet for analysis.

The monoterpenes were analysed with a 6890 N Network GC system coupled to a 5973 N MS (Agilent Technologies, Santa Clara, CA, USA) with a 30.0 m × 250 µm i.d. × 0.25 µm ZB-WAX column (Phenomenex). Sample injection volume was 1 µL in split/splitless mode (split ratio 10:1) at a temperature of 230°C. The temperature program was as follows: 40° C, held for 2 min, followed by 4°C/min to 220°C and held for 15 min. The sample was injected with an MPS 2 autosampler (Gerstel, Mühlheim, Germany). The carrier gas was helium with a constant flow of 1.3 mL/min. The temperature of the interface and MS source was 280 and 230°C, respectively. Data were acquired in full-scan mode (*m/z* 30–300). A more detailed description of the method has been published by Nitsch (2013). Monoterpenes were quantified using peak area of total ion current and a response factor calibration with the respective standard solutions of monoterpenes (Table S5). Analytical standards of pyran linalool oxides and diendiol 1 were not available.

A response factor (Rf) of 1 was used for these compounds [unit of measurement: µg/(L · Rf)]. All measurements were in duplicate.

Juice analysis

The concentration of malic acid, total acidity, alpha-amino acids (NOPA) and glucose + fructose was analysed by Fourier transform infrared spectroscopy. Spectra were recorded on an OenoFoss spectrometer (Foss Electric, Hillerød, Denmark) at ambient temperature. Spectra were exported to Matlab and analysed using an in-house partial least squares calibration.

Statistical analysis

Shading has been speculated to delay ripening and sugar accumulation in grapes. Delayed ripening has been speculated to influence terpenoid accumulation (Luan et al. 2006), and sugar accumulation alters gene expression of phenylpropanoid pathway genes (Ferri et al. 2011). To correct for eventual effects of differing sugar concentration between samples and sample groups, shade and control samples were compared using a two-factorial analysis of covariance (ANCOVA) with factors sampling date, treatment and sugar concentration (°Brix) as a covariate. The effect of reillumination was tested against permanently shaded samples using a one-factorial ANCOVA with sugar concentration (°Brix) as a covariate for every sampling date. All statistical calculations were conducted using SPSS software (IBM, Armonk, NY, USA).

Results and discussion

Studies with approaches to artificial bunch shading have been criticised, as bunch shading appears to delay grape ripening and therefore terpenoid accumulation (Luan et al. 2006). This comment may be expanded to several other studies focusing on microclimatic influence on terpenoid accumulation, in which sugar concentration was either decreased by shading (Reynolds and Wardle 1989) or not reported (Belancic et al. 1997). In contrast, several studies employing various bunch shading methodologies showed no effect or only a minor effect on grape ripening (Downey et al. 2004, Cortell and Kennedy 2006, Skinkis et al. 2010).

Sugars and organic acids

Sugar accumulation showed a consistent trend towards lower values in the shade and shade/light samples (Table 2). Total acidity and malic acid were both elevated in the shaded samples, except for the second sampling date. We chose a reillumination approach and utilised ANCOVA with sugar as a covariate for statistical evaluation to dispel doubt about the influence of light on the *V. vinifera* terpenoid metabolism as distinct from its developmental regulation.

Light influences terpenoid metabolism and monoterpene accumulation

Free monoterpenes were found in much smaller quantity than glycosylated monoterpenes (30% of monoterpenes in the control samples), with the exception of 3,7-dimethylocta-1,5-dien-3,7-diol (diendiol 1). Diendiol 1, a precursor of nerol and hotrienol and a typical terpenoid of Riesling-type cultivars (Rapp and Knipser 1979), was present at higher content in the free compared with that of the glycosylated fraction (Table 3). Our data clearly support the hypothesis that sunlight exposure increases terpenoid content in grapes. The content of all free monoterpenes, with the exception of free linalool, which just reached a detectable level in the control samples at harvest, was significantly elevated in the control as compared with that of the permanently shaded treatment. Twenty days after

Table 2. Effect of shading and light treatments on the composition and mass of Riesling berries at three periods during grape ripening.

	06/09/2012 (86 DAF)		20/09/2012 (100 DAF)			09/10/2012 (119 DAF)			Sig. °Brix	Sig. date	Sig. shading	Sig. light 8 h/20 days
	Control	Shade	Control	Shade	8 h light	Control	Shade	20 days light				
Berry mass (g)	1.23 ± 0.65	1.16 ± 0.15	1.38 ± 0.04	1.31 ± 0.07	1.29 ± 0.14	1.52 ± 0.04	1.40 ± 0.02	1.42 ± 0.04	*	n.s.	n.s.	n.s./n.s.
Sugars (°Brix)	13.88 ± 0.58	11.85 ± 1.79	15.8 ± 0.86	13.64 ± 0.8	14.09 ± 1.39	18.09 ± 0.48	16.2 ± 1.62	17.53 ± 1.25	—	***	***	n.s./n.s.
Titratable acidity (g/L)†	12.49 ± 0.21	15.14 ± 1.42	10.49 ± 0.54	10.94 ± 0.69	11.98 ± 1.2	7.55 ± 0.71	8.98 ± 0.24	8.48 ± 0.59	*	***	*	n.s./n.s.
pH	2.89 ± 0.03	2.85 ± 0.04	2.91 ± 0.05	2.93 ± 0.06	2.92 ± 0.05	3.04 ± 0.04	2.99 ± 0.06	3.05 ± 0.04	*	n.s.	n.s.	n.s./n.s.
Malic acid (g/L)	6.42 ± 0.24	8.08 ± 1.12	4.97 ± 0.37	5.53 ± 0.49	6.04 ± 1.16	3.37 ± 0.28	4.15 ± 0.3	4.07 ± 0.18	**	***	n.s.	n.s./n.s.

Data are the mean ± standard deviation (raw values). The effect of permanent shading was determined by a two-factorial ANCOVA with factors sampling date, treatment and sugar concentration (°Brix) as a covariate. The effect of reillumination was tested against permanently shaded samples using a one-factorial ANCOVA with sugar concentration (°Brix) as a covariate for every sampling date. *, **, ***, significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$. †Expressed as tartaric acid. Control, bunches permanently exposed to light; DAF, days after flowering; n.s., not significant; Shade, sunlight permanently excluded from bunches by sheltering in lightproof boxes; 8 h light and 20 days light, bunches shaded until the morning of 20 September 2012 and then exposed to light by removing boxes.

Table 3. Effect of shading and light treatment on the content of free and glycosylated monoterpenes in Riesling berries at three periods during grape ripening.

	Content (ng/berry)											
	06/09/2012 (86 DAF)		20/09/2012 (100 DAF)			09/10/2012 (119 DAF)			Sig. °Brix	Sig. date	Sig. shading	Sig. light 8 h/20 days
	Control	Shade	Control	Shade	8 h light	Control	Shade	20 days light				
Free monoterpenes												
trans-Linalool oxide furanoid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	—	—	—	—
cis-Linalool oxide furanoid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	—	—	—	—
Linalool	n.d.	n.d.	n.d.	n.d.	n.d.	22 ± 17	n.d.	n.d.	n.s.	n.s.	n.s.	—
Geraniol†	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	—	—	—	—
α-Terpineol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	—	—	—	—
trans-Linalool oxide pyranoid‡	71 ± 19	n.d.	90 ± 28	n.d.	n.d.	84 ± 22	n.d.	27 ± 18	n.s.	n.s.	***	—/n.s.
cis-Linalool oxide pyranoid‡	33 ± 9	n.d.	65 ± 61	n.d.	n.d.	n.d.	n.d.	n.d.	n.s.	n.s.	**	—
3,7-dimethylocta-1,5-dien-3,7-diol‡	299 ± 51	60 ± 16	338 ± 79	49 ± 12	43 ± 8	667 ± 87	43 ± 9	67 ± 14	n.s.	**	***	n.s./*
Total free monoterpenes	403 ± 77	60 ± 16	495 ± 56	49 ± 12	43 ± 8	773 ± 112	43 ± 9	95 ± 32	n.s.	*	***	n.s./n.s.
Glycosylated monoterpenes												
trans-Linalool oxide furanoid	65 ± 8	n.d.	106 ± 35	n.d.	n.d.	230 ± 65	n.d.	36 ± 24	n.s.	*	***	—/*
cis-Linalool oxide furanoid	85 ± 22	64 ± 14	119 ± 44	86 ± 17	72 ± 20	78 ± 21	62 ± 11	62 ± 14	n.s.	*	n.s.	n.s./n.s.
Linalool	106 ± 39	n.d.	188 ± 54	n.d.	n.d.	862 ± 215	n.d.	36 ± 8	n.s.	***	***	—/***
Geraniol	114 ± 37	73 ± 22	118 ± 24	85 ± 33	87 ± 19	121 ± 25	88 ± 13	97 ± 22	n.s.	n.s.	*	n.s./n.s.
α-Terpineol	61 ± 12	46 ± 9	62 ± 13	43 ± 4	43 ± 12	75 ± 10	54 ± 5	62 ± 5	n.s.	n.s.	*	n.s./n.s.
trans-Linalool oxide pyranoid‡	n.d.	n.d.	15 ± 17	n.d.	n.d.	58 ± 18	n.d.	n.d.	n.s.	*	***	—
cis-Linalool oxide pyranoid‡	30 ± 34	n.d.	77 ± 37	45 ± 15	27 ± 18	56 ± 16	19 ± 23	38 ± 33	n.s.	**	*	n.s./n.s.
3,7-dimethylocta-1,5-dien-3,7-diol‡	83 ± 19	n.d.	134 ± 46	n.d.	n.d.	418 ± 120	n.d.	40 ± 9	n.s.	*	***	n.s./***
Total glycosylated monoterpenes	544 ± 120	182 ± 42	819 ± 225	259 ± 50	229 ± 48	1899 ± 412	224 ± 39	370 ± 83	n.s.	**	***	n.s./*

Data are the mean ± standard deviation (raw values).The effect of permanent shading was determined by a two-factorial ANCOVA with factors sampling date, treatment and sugar concentration (°Brix) as a covariate. The effect of reillumination was tested against permanently shaded samples using a one-factorial ANCOVA with sugar concentration (°Brix) as a covariate for every sampling date.*, **, ***, significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$. †Free geraniol was detectable, but not quantified because of co-elution with a second peak; ‡Unit of measurement: ng/(berry · response factor). Control, bunches permanently exposed to light; DAF, days after flowering; n.d., not detected; n.s., not significant; Shade, sunlight permanently excluded from bunches by sheltering in lightproof boxes; 8 h light and 20 days light, bunches shaded until the morning of the 20 September 2012 and then exposed to light by removing boxes.

reillumination, the content of free *cis*-linalool oxide pyranoid and diendiol 1 was significantly elevated compared with that of the shaded treatment but significantly lower than that of the control samples ($P < 0.05$).

A strong increase in the content of glycosylated linalool, *cis*-linalool oxide furanoid, *cis*-linalool oxide pyranoid and diendiol 1 was recorded between sampling dates. The content of *cis*-linalool oxide furanoid, geraniol and α-terpineol was affected to a minor extent only by shading and did not increase significantly during the sampling period (Table 3). Therefore, it appears likely that the synthesis of these compounds virtually ceases shortly after veraison. Future studies will aim to investigate the effect of light on the metabolism of these compounds during an earlier stage of ripening. Constant shading almost completely inhibited monoterpene accumulation, leading to a 90% reduction of monoterpene content at harvest. The strongest effect was observed for linalool and diendiol 1 (Figure S3), which were not detected in shaded bunches, but which were present in high content in the control treatment. Reillumination 20 days before harvest increased glycosylated monoterpenes by 77% compared with that of shaded bunches.

At harvest, the mean concentration of monoterpenes was 190, 325 and 1753 µg/L for shade, 20 days light and control berries, respectively (Table S6). Similar values for monoterpene and especially linalool concentration were reported for the same Riesling clone (Gm 198-25) in a similar location (Hey et al. 2008).

Our data agree with the results of Zoecklein et al. (1998), who showed that increased sun exposure through defoliation increased glycosylated terpenoids in Riesling even at lower sugar concentration. Reynolds and Wardle (1989) found significantly increased concentration of glycosylated terpenoids, but little difference in free terpenoid concentration between exposed and semi-shaded as well as shaded bunches in Gewürztraminer. They further noted a tendency towards a lower concentration of free terpenoids in severely shaded bunches. Furthermore, our data agree with the results obtained by Belancic et al. (1997), who found a decreased concentration of free and glycosylated monoterpenes in severely shaded bunches as compared with that in sunlit and semi-shaded bunches on Moscatel Rosado and Muscat of Alexandria cultivars. Interestingly, and in agreement with our study, the monoterpenes most affected by sunlight exposure in the latter study were linalool and other linalool-derived monoterpenes.

Our data, however, only partially agree with that published by Skinkis et al. (2010), who found that canopy shading decreases glycosylated but increases free terpenoids in the interspecific hybrid Traminette. Data from other studies suggest that terpenoid accumulation is not influenced or even reduced by solar radiation (Bureau et al. 2000a, Scafidi et al. 2013) or decreased by severe artificial shading but increased by canopy shading (Bureau et al. 2000b). One explanation for these different observations is the climatic conditions under which these studies were conducted: while the study of Reynolds et al. (1989) and our study were conducted in a cool climate (Ontario, Canada and Central Germany), the data of Skinkis et al. (2010), Bureau et al. (2000a, 2000b) and Scafidi et al. (2013) were obtained in warmer climates (Southern Indiana, USA; the Languedoc region, France; and Sicily, Italy), which favour the volatilisation of terpenoids, especially when berries are heated additionally by exposure to direct sunlight. Hence, increasing light exposure of berries, for example, by leaf removal, appears to be beneficial for grape aroma in cooler climates. In hot climates, the beneficial effect of increased synthesis of terpenoids induced by light may be surpassed by the negative effect of elevated berry temperature, that is, increased loss by volatilisation. It needs to be stated that, because of the elevated temperature of exposed berries, it cannot be excluded that temperature effects on berry metabolism occurred in this study. Elevated berry temperature, however, appears to lead to reduced terpenoid accumulation (Scafidi et al. 2013). Further, optimum temperature for 1-deoxy-D-xylulose 5-phosphate synthase activity has been shown to be at 37°C (Battilana et al. 2011), a temperature easily surpassed in exposed berries even in cool–moderate climates (Stoll and Jones 2007).

The strong effect of shading in our study as compared with that in other studies might further be explained by the severity of the applied shading treatment. While shaded bunches in our study received only 6.5 µmol/(s · m^2) or less than 1% of total ambient, photosynthetically active, photon flux density (Figure S1), the shaded bunches in the study of Belancic et al. (1997) still received 125 µmol/(s · m^2) or 19% of photosynthetically active, photon flux density around solar noon.

Expression of the two linalool/nerolidol synthase genes *VvTPS54* (*VvPNLinNer1*) and *VvTPS56* (*VvPNLinNer2*) found in Cabernet Sauvignon and Pinot Noir was confirmed in Riesling, while expression of *VvTPS61* (*VvPNLGl4*) was not detected in Riesling using the primer pair as published for Pinot Noir by Martin et al. (2012). The enzymes TPS54 and TPS56 produce linalool from geranyl pyrophosphate and nerolidol from farnesyl pyrophosphate (Martin et al. 2010) and are therefore highly relevant for Riesling aroma. The expression of *VvTPS54* and *VvTPS56* was significantly lower in shaded berries than in control berries at all three sampling dates. After 8 h of sun exposure, *VvTPS54* and *VvTPS56* expression increased significantly ($P < 0.05$) compared with that of the shaded berries. After 20 days of sun exposure, *VvTPS54* expression was higher than that in shaded berries ($P < 0.001$) but still significantly lower than that of control berries. Expression of *VvTPS54* was highest 20 days before harvest and declined slightly afterwards. In contrast to *VvTPS54* expression, *VvTPS56* expression declined steadily over the three sampling dates in shaded as well as exposed berries (Figure 2). At harvest, *VvTPS56* expression in berries exposed 20 days was not significantly different from that of the control berries. No study to date has confirmed the activation of terpenoid synthase genes in grapes by illumination; however, UV-B light increased *VvTPS* gene expression in grapevine leaves by 1.9-fold to 2.6-fold (Pontin et al. 2010) and VvTPS activity by twofold to eightfold, with low but constant UV-B irradiation showing a stronger effect than a short-time, high-dose UV-B radiation (Gil et al. 2012).

(*E*)-4-Hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR) is a key enzyme in the MEP pathway, catalysing the branching of (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate to IPP and DMAPP; *VvHDR* transcript accumulation appears to correlate well with terpenoid accumulation (Martin et al. 2012). In our study, *VvHDR* gene expression did not change significantly during the experimental period. No influence of light on *VvHDR* expression was observed, although light appeared to promote *VvHDR* expression during the first two sampling dates. Accumulation of *VvHDR* transcript did not correlate significantly with the accumulation of linalool or other monoterpenes. In contrast, *VvTPS54* gene expression correlated with the accumulation of free and glycosylated monoterpenes, and *VvGT56* gene expression correlated weakly with the accumulation of free monoterpenes (Table S4). It appeared, however, that maximum linalool accumulation took place a few weeks after the peak of linalool biosynthetic gene expression (Figure 2). This is in accordance with Matarese et al. (2013),

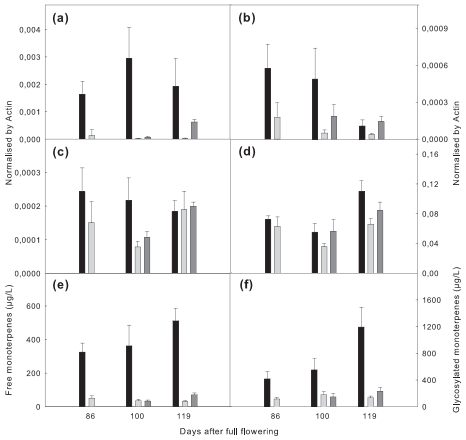

Figure 2. Effect of illumination regime on the expression of the genes (a) *VvTPS54*, (b) *VvTPS56* and (c) *VvHDR* and (d) the monoterpene glucosyltransferase *VvGT7* and on the concentration of (e) total free and (f) glycosylated monoterpenes in Riesling grapes during ripening. The illumination regimes were as follows: light (control), bunches exposed to ambient sunlight, as found in a regular vertical shoot position canopy (■); shade, bunches completely sheltered from light from 60% veraison [66 days after full flowering (DAF)] until harvest (119 DAF) (■); and shade/light, bunches shaded until 20 days before harvest (100 DAF) and then exposed to sunlight (■). All data represent mean ± standard deviation of the raw values. Effect of permanent shading was determined by a two-factorial ANCOVA with factors sampling date, treatment and sugar concentration (°Brix) as a covariate. Effects of reillumination were tested against permanently shaded samples using a one-factorial ANCOVA with sugar concentration (°Brix) as a covariate for every sampling date.

who have shown that the maximum daily increment in linalool/geraniol content occurs about 2 weeks after the peak linalool/geraniol synthase expression, which occurs 20–30 days post-veraison in cv. Moscato Bianco. It is, therefore, likely that terpenoid synthases undergo significant post-translational modification in order to gain their full functionality. Expression of all linalool synthase genes appears to decline towards harvest (Martin et al. 2012, Matarese et al. 2013), which may explain the rather small increment in terpenoid content by reillumination during the last weeks of ripening in our study.

The GTs, VvGT7, VvGT14 and VvGT15, are the only published GTs in *V. vinifera* showing uridine diphosphate (UDP)-glucose transfer activity towards monoterpenols (Bönisch et al. 2014a, 2014b). Statistical analysis revealed that *VvGT7* transcript accumulation was related to development and light ($P < 0.05$, Figure 2) and that it correlated significantly with the accumulation of glycosylated monoterpenes (Table S4). Expression reached a minimum 3 weeks before harvest and rose thereafter (Figure 2), which is in accordance with the results presented by Bönisch et al. (2014b). In their study, however, *VvGT7* expression did not appear to be correlated with terpenoid accumulation. Expression of two further terpenoid GTs, *VvGT14* and *VvGT15* (Bönisch et al. 2014a), was not influenced by light (Figure S2). The expression of additional GTs, with to date unknown substrate specificities, has been investigated with GeXP. Of these GTs, *VvGT12* (Bönisch et al. 2014a) was influenced by light ($P < 0.001$) and correlated well with *VvTPS* and *VvHDR* gene expression. The enzyme VvGT12 did not glucosylate any of the tested phenolic or terpenoid substrates in the study of Bönisch et al. (2014b). It has to be noted, however, that sugar substrates other than UDP-glucose have not been tested in this study. Therefore, this enzyme may transfer a different sugar than glucose to an already glucosylated terpenoid or transfer a diglycoside. More research on the terpenoid GTs using different sugar donors is therefore necessary to elucidate their metabolic role.

Flavonol metabolism shows a stronger dependency on light than terpene metabolism

A significant difference was observed for flavonols between shaded and exposed bunches on the first and second sampling date, while 8 h of light exposure did not lead to a measurable increase of phenolic substances (Table 4). At maturity, after 20 days of light exposure, a significant increase was observed for all quercetin (que) glycosides. Quercetin-3-glucuronide, que-3-galactoside, que-3-rutenoside, que-3-xyluloside and que-3-glucoside content was lower after 20 days of exposure compared with that of the control, while the content of que-3-arabinoside and que-3-rhamnoside was higher. Data collected at the same experimental site in various experiments suggest that the latter flavonols are accumulated mainly during the late ripening stage (Matthias Friedel, unpubl. data, 2012). Quercetin glycosides are the main flavonols of Riesling, comprising 85% of total flavonols present (Mattivi et al. 2006). Riesling also contains a significant amount of kaempferol glycosides and a small amount of isorhamnetin glycosides (12.5 and 2.7% of flavonols, respectively). These substances were not detected with our method; however, a similar light-dependent effect on these substances can be expected as their synthesis also depends on *VvFLS1*. The content of hydroxycinnamates and flavanols remained unaffected by the treatments.

Relative expression of *VvFLS1*, the key enzyme in flavonol metabolism, was virtually zero in shaded treatments at all sampling dates during the ripening phase (Figure 3). In the control treatment, FLS expression was constant between the 6 and 20 September 2012 samplings, but increased at ripeness. After receiving light for about 8 h, the mean relative expression of *VvFLS1* increased significantly ($P < 0.05$) from 2.5×10^{-5} to 1.2×10^{-4}. In cell cultures, *FLS* gene expression is induced even faster and to a larger extent (Czemmel et al. 2009), possibly because light penetration into a cell culture sample is more complete than into a grape bunch. Twenty days after reillumination, *VvFLS1* expression was higher than that in the control treatment. This may be explained by higher light exposure of bunches that were previously sheltered in boxes. Standard errors in the field replicates were low, and an influence of sugar concentration on the expression of *FLS* genes, as observed for several genes of the phenylpropanoid pathway by Ferri et al. (2011), was not detected. The expression of *VvFLS1* showed a strong and direct correlation to the accumulation of flavonols ($r = 0.91$, Table S4). Even variance of *VvFLS1* expression within a treatment, maybe originating from a small difference in the degree of illumination, was reflected as a variance in the flavonol content. Matus et al. (2009) showed a similar reaction of flavonol metabolism in Cabernet Sauvignon. In their study, *VvFLS1* and *VvMYB12* (identical with *VvMYBF1*) expression in a delayed exposure treatment surpassed expression of these genes in the control treatment at harvest. The content of flavonols, however, in the delayed exposure treatment in their study was lower than that of the control at harvest. This might be explained by the fact that the last sampling for flavonols in their study was conducted only 2 weeks after reillumination of these samples, compared with 20 days in our study.

The GTs *VvGT5* and *VvGT6* catalyse the glycosylation of quercetins, producing que-3-glucuronide, que-3-galactoside

Table 4. Effect of shading and light treatment on the content of phenolic substances in Riesling berries at three periods during grape ripening.

	Content (µg/berry)										
	06/09/2012 (86 DAF)		20/09/2012 (100 DAF)			09/10/2012 (119 DAF)			Sig. °Brix	Sig. date	Sig. shade	Sig. light 8 h/20 days
	Control	Shade	Control	Shade	8 h light	Control	Shade	20 days light				
Flavanols												
Procyanidin B1	1.9 ± 0.7	2.4 ± 0.8	1.9 ± 0.9	2.4 ± 0.4	1.5 ± 0.5	1.6 ± 1.7	0.4 ± 0.3	1.2 ± 1.3	n.s.	n.s.	n.s.	*/n.s.
Catechin	21.1 ± 6.2	24.8 ± 7.6	33.3 ± 24.4	19.0 ± 2.5	17.8 ± 6.6	15.1 ± 7.5	14.5 ± 2.7	18.7 ± 12.5	n.s.	n.s.	n.s.	n.s./n.s.
Procyanidin B2	152.3 ± 51.0	118.4 ± 71.3	66.2 ± 19.8	62.4 ± 18.8	68.2 ± 41.3	49.8 ± 17.8	68.7 ± 6.0	59.6 ± 25.1	n.s.	**	n.s.	n.s./n.s.
Epicatechin	35.1 ± 10.0	42.0 ± 7.0	47.8 ± 24.8	45.2 ± 6.2	43.3 ± 17.2	35.7 ± 7.3	32.8 ± 19.8	39.0 ± 8.2	n.s.	n.s.	n.s.	n.s./n.s.
Total flavanols	210.3 ± 67.1	187.6 ± 73.0	149.2 ± 33.1	129.0 ± 19.2	130.8 ± 64.6	102.2 ± 23.5	116.4 ± 15.2	118.6 ± 44.3	n.s.	n.s.	n.s.	n.s./n.s.
Hydrocinnamates												
Glutathionyl-caftaric acid	4.0 ± 0.3	3.7 ± 1.3	6.0 ± 0.6	6.0 ± 1.2	5.5 ± 0.8	10.1 ± 1.2	11.3 ± 0.9	11.1 ± 1.6	**	***	n.s.	n.s./n.s.
Cumaroylglucose	3.0 ± 0.3	2.2 ± 0.3	3.6 ± 1.3	2.2 ± 0.5	1.8 ± 0.6	2.5 ± 1.6	2.1 ± 0.2	2.3 ± 1.3	n.s.	n.s.	n.s.	n.s./n.s.
Caftaric Acid	104.9 ± 5.1	113.7 ± 7.9	120.7 ± 16.6	111.0 ± 6.9	87.5 ± 38.2	83.4 ± 35.6	108.4 ± 11.2	89.0 ± 30.7	n.s.	n.s.	n.s.	n.s./n.s.
Coutaric acid	23.8 ± 2.3	33.0 ± 5.3	29.3 ± 8.6	28.0 ± 2.6	20.7 ± 13.5	19.6 ± 11.1	28.1 ± 2.5	19.6 ± 7.4	n.s.	n.s.	n.s.	n.s./n.s.
p-Coumaroylglucosyl-tartrate	1.3 ± 0.1	1.6 ± 0.2	1.4 ± 0.2	1.4 ± 0.3	1.3 ± 0.1	1.2 ± 1.0	1.5 ± 0.2	1.5 ± 0.8	n.s.	n.s.	n.s.	n.s./n.s.
Fertaric acid	9.0 ± 0.5	10.5 ± 0.4	10.1 ± 0.9	9.8 ± 1.0	8.6 ± 1.5	9.6 ± 1.0	10.2 ± 1.1	9.5 ± 0.4	n.s.	n.s.	n.s.	n.s./n.s.
Caffeic acid	2.0 ± 0.2	1.2 ± 0.1	2.5 ± 1.8	1.5 ± 0.3	1.6 ± 0.4	2.3 ± 0.6	1.4 ± 0.3	1.9 ± 0.6	n.s.	n.s.	n.s.	n.s./n.s.
Total hydroxycinnamates	147.9 ± 7.3	165.7 ± 12.1	173.6 ± 27.0	159.6 ± 11.6	126.8 ± 53.4	128.6 ± 49.3	163.0 ± 13.8	134.9 ± 40.1	n.s.	n.s.	n.s.	n.s./n.s.
Flavonol glycosides												
Que-3-rutenoside	7.7 ± 1.3	3.6 ± 1.3	6.4 ± 1.4	2.3 ± 0.6	3.1 ± 1.5	6.8 ± 1.0	2.8 ± 0.6	4.9 ± 1.5	n.s.	n.s.	n.s.	n.s./n.s.
Que-3-galactoside	3.5 ± 0.8	0.6 ± 0.3	3.6 ± 0.7	0.4 ± 0.1	0.5 ± 0.2	5.9 ± 0.3	0.5 ± 0.1	4.5 ± 0.8	n.s.	**	***	n.s./***
Que-3-glucoside	12.6 ± 3.4	1.2 ± 0.4	13.2 ± 2.4	1.0 ± 0.1	1.1 ± 0.4	22.4 ± 1.7	1.2 ± 0.3	18.7 ± 3.1	n.s.	**	***	n.s./***
Que-3-glucuronide	19.8 ± 1.3	10.6 ± 3.1	17.1 ± 4.6	6.8 ± 2.0	8.0 ± 4.0	17.1 ± 3.1	7.0 ± 1.7	12.9 ± 4.0	n.s.	n.s.	n.s.	n.s./n.s.
Que-3-xyloside	0.5 ± 0.1	0.3 ± 0.1	0.5 ± 0.4	0.1 ± 0.1	0.1 ± 0.2	0.7 ± 0.4	n.d.	0.4 ± 0.1	n.s.	n.s.	n.s.	n.s./**
Que-3-arabinoside	1.0 ± 0.3	0.2 ± 0.1	2.4 ± 0.8	0.1 ± 0.1	0.2 ± 0.2	3.6 ± 0.8	0.1 ± 0.1	6.2 ± 1.0	n.s.	n.s.	***	n.s./***
Que-3-rhamnoside	4.1 ± 1.7	0.3 ± 0.1	5.7 ± 0.8	0.2 ± 0.1	0.1 ± 0.1	12.0 ± 1.7	0.1 ± 0.1	25.2 ± 4.9	n.s.	***	***	n.s./***
Total flavonol glycosides	49.2 ± 8.3	16.8 ± 5.3	48.8 ± 9.0	10.9 ± 2.9	13.2 ± 6.2	68.4 ± 6.7	11.6 ± 2.8	72.8 ± 12.3	n.s.	n.s.	**	n.s./***

Data are the mean ± standard deviation (raw values). The effects of permanent shading were determined by a two-factorial ANCOVA with factors sampling date, treatment and sugar concentration (°Brix) as a covariate. The effects of reillumination was tested against permanently shaded samples using a one-factorial ANCOVA with sugar concentration (°Brix) as a covariate for every sampling date. *, **, ***, significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$. Control, bunches permanently exposed to light; DAF, days after flowering; n.s., not significant; Shade, sunlight permanently excluded from bunches by sheltering in lightproof boxes; 8 h light and 20 days light, bunches shaded until the morning of the 20 September 2012 and then exposed to light by removing boxes.

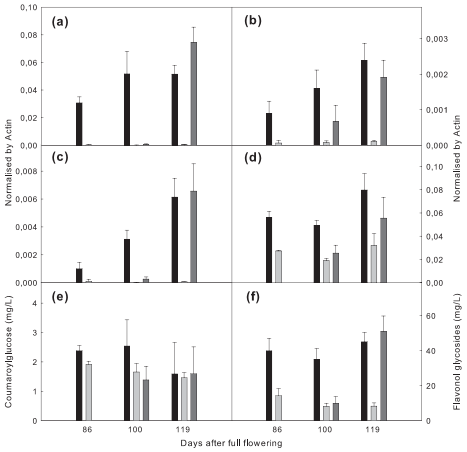

Figure 3. Effect of illumination regime on the expression of the genes (a) *VvFLS1*, (b) *VvGT5*, (c) *VvGT6* and (d) *VvGT9* and on the concentration of (e) coumaroylglucose and (f) flavonol glycosides. The illumination regimes were as follows: light (control), bunches exposed to ambient sunlight, as found in a regular vertical shoot position canopy; shade, bunches completely sheltered from light from 60% veraison [66 days after full flowering (DAF)] until harvest (119 DAF) (▫); shade/light: bunches shaded until 20 days before harvest (100 DAF) and then exposed to sunlight (▪). All data represent mean ± standard deviation of raw values. Effects of permanent shading were determined by a two-factorial ANCOVA with factors sampling date, treatment and sugar concentration (°Brix) as a covariate. Effects of reillumination were tested against permanently shaded samples using a one-factorial ANCOVA with sugar concentration (°Brix) as a covariate for every sampling date.

and que-3-glucoside (Ono et al. 2010). Their expression is speculated to be coordinately controlled by common transcription factors (Ono et al. 2010) such as VvMYBF1, which has been shown to control *VvFLS1* transcription (Czemmel et al. 2009). A clear influence of light on gene expression was observed for *VvGT5* and *VvGT6*, as the expression of *VvGT5* and *VvGT6* genes increased significantly after 8 h of insolation ($P < 0.05$). The expression of *VvGT6* was influenced more by insolation, with virtually no expression in the shaded treatment, and equal transcript levels in control and 20-day insolation treatment at harvest (Figure 3). Expression of *VvGT5* was low, but detectable in the shaded treatment. In our study, expression of these two GTs was strongly intercorrelated, especially at full maturity. Expression of both *GT* genes was further correlated to *VvFLS1* gene expression (Table S4), as well as to the accumulation of que-3-glucoside and que-3-galactoside, the products of *VvGT6*. They were not correlated to the accumulation of que-3-glucuronide, the product of *VvGT5* (data not shown). Of the three phenolic acid GT genes, *VvGT9*, *VvGT10* and *VvGT11* (Khater et al. 2011), expression of only *VvGT9* was influenced by light ($P < 0.05$), while *VvGT10* and *VvGT11* were not influenced or not expressed, respectively (Figures 3 and S2). The accumulation of hydroxycinnamic acid derivates was not correlated to phenolic acid *GT* gene expression (Table S4), probably because the hydroxycinnamate content of the berries remained constant over the investigated time span.

Additionally, the expression of seven *VvGTs (VvGT8, VvGT13, VvGT16 to VvGT20)*, of which the natural substrates have not been identified so far, was analysed by GeXP (Figure S2). Transcripts of *VvGT8*, *VvGT17* and *VvGT20* were not detected in all samples, as in Bönisch et al. (2014a, 2014b), who showed that transcription of these *GT* genes is almost absent after veraison. Nevertheless, transcripts were detected more frequently in sun-exposed samples. The expression of *VvGT19* was down-regulated by light ($P < 0.01$). The remaining *VvGTs* were not affected by light (*VvGT13*, *VvGT16* and *VvGT18*).

Comparison of the metabolism of phenolic substances and terpenoids

A comparison of the metabolism of phenolic substances and terpenoids revealed that the expression of *VvHDR*, *VvTPS* and *VvGT7* genes is activated slower and to a lower extent in response to light than that of the flavonoid pathway genes or is even not affected at all (*VvGT14* and *VvGT15*). In contrast, the expression of genes of flavonol metabolism is strongly influenced by light, especially during the last weeks of ripening. This was also reflected in the rapid accumulation of flavonoid metabolic products upon reillumination, while only a much smaller effect was observed on terpenoid accumulation (Figures 1 and 2). Similarly, transcripts of the flavonol GTs *VvGT5* and *VvGT6* were virtually absent in shaded bunches and increased instantly after reillumination. In contrast, all examined terpenoid GTs were expressed in shaded bunches, and no (*VvGT13* and *VvGT14*) or only little (*VvGT7*) influence of light was observed on their expression. It therefore appears likely that flavonol synthesis including flavonol GT gene expression is coordinately controlled by a light-induced transcription factor such as VvMYBF1, while terpenoid GT expression may be rather developmentally regulated. Other terpenoid GTs, however, which have not been identified yet, could be light inducible and responsible for glycosylation of monoterpenes upon illumination. No light-induced transcription factor for terpenoid metabolism has been reported so far, and therefore, more research is necessary to elucidate whether monoterpene metabolism is controlled by such a transcription factor and if there is a common control of monoterpene metabolic gene expression. Future studies should also include the analysis of enzymes located early in the MEP pathway, as some of these, in particular 1-deoxy-D-xylulose 5-phosphate synthase, appear to play an important role in terpenoid accumulation (Battilana et al. 2011).

Although control berries and those subjected to 20-day light treatment were almost equal in their content of phenolic substances, total monoterpene content was less than 20% of the control samples in the 20-day light treatment. This difference can be attributed to the decreased expression of *VvTPS* genes towards harvest, which is in accordance with other studies on grape berries (Martin et al. 2012, Matarese et al. 2013). Furthermore, it has been shown that UV-B radiation increases VvTPS activity and terpenoid accumulation in grapevine leaves, with young tissue showing a stronger response (Gil et al. 2012). Terpenoid accumulation in leaves and berries may follow a similar pattern, with younger berry tissues showing a stronger response to light.

Although transcription of both flavonol and terpenoid metabolic genes is induced by light, it appears likely that both pathways are regulated differentially. For instance, jasmonate synthesis and jasmonate-induced defence reactions have been shown to be down-regulated under shade conditions (Agrawal et al. 2012). Application of methyl jasmonate increases *VvTPS* gene expression (Fäldt et al. 2003), as well as terpenoid accumulation in grapes (D'Onofrio et al. 2009, Gómez-Plaza et al. 2012) and other plants (Martin et al. 2003, de la Peña Moreno et al. 2010). Methyl jasmonate application, however, does not increase flavonol accumulation in grape (Ruiz-García et al.

2013) and other fruit (de la Peña Moreno et al. 2010). Therefore, the lower monoterpene content under shade conditions may be associated with a lower activity of jasmonate under these conditions. Flavonol metabolism appears to be regulated in a more direct, specific and rapid way in reaction to changing light environment. Utilising sugar concentration as a covariate in the data analysis showed that in contrast to the concentration of quantitatively important compounds such as organic acids, neither gene expression of monoterpenes and flavonol biosynthetic genes nor the accumulation of their respective products was significantly influenced by sugar concentration of the berries.

Flavonol accumulation precisely matched *VvFLS1* gene expression, while monoterpene accumulation appeared to reach its peak about 2 weeks after maximum *VvTPS* gene expression. Although flavonol and terpenoid metabolism are apparently regulated by different signal cascades, a study on snapdragon (Dudareva et al. 2003) and a recent study on tomato (Kang et al. 2014) revealed a potential crosstalk between flavonol and terpenoid metabolism. In the latter study, a chalcone isomerase-deficient mutant, strongly inhibited in flavonoid, especially flavonol, production, also produced much less terpenoids than control plants. The authors of the study speculate that an intermediate or end product of flavonol metabolism may promote terpenoid synthesis. This hypothesis might explain that, while both *VvTPS* and *VvFLS* transcription were induced after 8 h of insolation, monoterpenes accumulated much slower than flavonols. While Kang et al. (2014) also deem the alternative hypothesis possible, that is, that terpenoid synthesis may be inhibited by the accumulation of phenylpropanoid metabolic intermediates, our data show that gene expression of both flavonol and terpenoid metabolic genes is simultaneously up-regulated by similar environmental conditions. Further, accumulation of flavonoids and monoterpenes takes place at the same developmental stages of the grape, flowering and ripening. Between flowering and ripening, only small amounts of monoterpenes and flavonoids are synthesised. We therefore speculate that intermediates or end products of flavonol metabolism may play a crucial role in the post-transcriptional regulation of the VvTPS enzyme synthesis.

Conclusion

In this study, we have shown that both monoterpene synthases and FLSs are simultaneously up-regulated by light influence during grape ripening. Similarly, the accumulation of end products of both metabolic pathways was positively influenced by light. Maximum synthesis of monoterpenes appears to occur about 2 weeks after maximum *VvTPS* gene expression, while flavonol accumulation occurred both more rapidly and to a larger extent upon light induction of *VvFLS1*. As a practical conclusion, increasing radiation interception through hedging or defoliation around veraison, when the expression of the *VvTPS-g* subfamily of genes reaches its maximum, may be much more beneficial to grape flavour development than at the final stage of ripening, when defoliation is often applied to manipulate the canopy microclimate because of phytosanitary reasons.

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Supporting information

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Figure S1. (a) Temperature inside a box as used in the experiments (——) compared with that of sensors beside the box (- - - -). (b) Radiation measured by photosynthetically active radiation (PAR) sensors placed inside the boxes (——) utilised for light exclusion, compared with ambient PAR (- - - -). PPFD, photosynthetically active, photon flux density.

Figure S2. Expression of all VvGT genes analysed by GeXP under different illumination regimes: light, bunches exposed to ambient sunlight, as found in a regular vertical shoot position canopy (■); shade, bunches completely sheltered from light from 60% veraison [66 days after flowering (DAF)] until harvest (119 DAF) (■); and shade/light, bunches shaded until 20 days before harvest (100 DAF) and then exposed to sunlight (■). Data are presented as mean ± standard deviation. All data represent raw values. Effects of permanent shading were determined by a two-factorial ANCOVA with factors sampling date, treatment and sugar concentration (°Brix) as a covariate. Effects of reillumination were tested against permanently shaded samples using a one-factorial ANCOVA with sugar concentration (°Brix) as a covariate for every sampling date.

Figure S3. Chromatogram of the monoterpenes of Riesling berries at harvest (119 days after full flowering). (a) Bunches exposed to light from veraison to harvest (control) and (b) bunches shaded from veraison to harvest. 1, internal standard, 2-octanol; 2, *trans*-linalool oxide furanoid; 3, *cis*-linalool oxide furanoid; 4, linalool; 5, α-terpineol; 6, *trans*-linalool oxide pyranoid; 7, *cis*-linalool oxide pyranoid; 8, geraniol; 9, diendiol 1.

Table S1. Primer sequences. Gene specific primers used for GeXP (GeXP_for and GeXP_rev) are chimeric and contain a universal tag sequence at their 5′-end (lowercase). Primers for quantitative RT-PCR (RT_for and RT_rev) are also specified.

Table S2. Final concentration of each reverse primer in the GeXP reverse transcription reaction.

Table S3. List of standards used for calibration of the HPLC method for the determination of grape skin phenolic substances.

Table S4. Correlation table of gene expression and the concentration of sugars (°Brix), hydroxycinnamates, flavonols and free and bound monoterpenes.

Table S5. Additional information on the GC/MS calibration.

Table S6. Effect of shading and light treatment on the concentration of free and bound monoterpenes in Riesling berries at three periods after flowering.

Chapter 5

Influence of berry diameter and colour on some determinants of wine composition of *Vitis vinifera* L. cv. Riesling[4]

[4]Friedel M., Sorrentino V., Blank, M. & Schüttler A. 2016. Influence of berry diameter and colour on some determinants of wine composition of *Vitis vinifera* L. cv. Riesling. *Australian Journal of Grape and Wine Research* **22** (2), 215-225

Influence of berry diameter and colour on some determinants of wine composition of *Vitis vinifera* L. cv. Riesling

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Abstract

Background and Aims: To successfully use berry sorting in winemaking, it is crucial to understand the interaction of physical and chemical composition of berries. The aim of this study was to investigate the relationship between berry diameter and colour and aspects of wine composition, such as titratable acidity, aroma compounds and phenolic substances of *Vitis vinifera* L. cv. Riesling.
Methods and Results: In a first trial, berries were sorted into three berry diameter classes with equal TSS concentration and vinified by 70 mL scale fermentation. In a second trial, berries from each of two diameter classes with equal TSS concentration were sorted by berry colour to obtain samples of low a* value and high a* value berries for the respective diameter class, and vinified. In the first trial, wines from smaller berries had lower titratable acidity and a lower concentration of malic acid. In the second trial, wine obtained from berries with higher a* values showed a higher concentration of free C_{13}-norisoprenoids as well as free and glycosylated monoterpenes. Wines from smaller berries in this trial showed a higher concentration of norisoprenoids and a lower pH.
Conclusions: Berry diameter and colour are highly variable within single vineyards, vines and single bunches. Sorting by berry size or colour will lead to wines with a pronounced difference in aroma compounds, acidity and α-amino nitrogen.
Significance of the Study: This study shows the relationship between berry diameter or colour and wine quality aspects such as acidity and aroma. Understanding this relationship will assist winemakers to conduct targeted berry sorting.

Keywords: *aroma, berry colour, berry diameter, optical sorting, Riesling, terpenoids*

Introduction

The extent to which spatial, systematic variability of grape composition is encountered in commercial vineyards has highlighted the potential benefits of precision viticulture (Letaief et al. 2008, Bramley et al. 2011a,b). It has also been shown that non-spatial variability of grape composition exists within a bunch and among bunches of individual vines (Kasimatis et al. 1975, Tarter and Keuter 2005). While reducing heterogeneity of the harvested grapes is assumed to be beneficial for wine quality (Keller 2010, Barbagallo et al. 2011), 'it is extremely difficult to obtain uniform berry diameter and composition under field conditions, even when all vineyard management practices are properly executed' (Pisciotta et al. 2013). As precision viticulture and selective harvesting can only address systematic variability of grape composition within single vineyards, postharvest berry sorting appears to be a promising technological approach to reduce variability of grape composition introduced by intra-vine and intra-bunch variations. It may further provide opportunities for the selection of grapes for the production of ultra-premium wines.

Berry diameter and colour are two parameters that are easily measured and that are highly variable in vineyards. Both parameters can be exploited in technical separation processes such as targeted berry sorting. In recent years, several companies have developed optical berry sorting machines [e.g. Bucher Vaslin and Pellenc; Pellenc and Niero (2014)], which are able to recognise object size, shape and reflectance in various regions of the electromagnetic spectrum, permitting the elimination of rotten berries and material other than grape (Falconer et al. 2006). This technology could therefore allow producers to create wines of variable composition from a single production unit in a targeted way. As berry diameter and colour do not, however, influence white wine composition per se, understanding the relationship between berry diameter and colour and wine composition is essential in order to benefit from sorting operations.

In viticulture, it is often assumed that wines produced from smaller berries will lead to higher quality wines because of a higher skin to pulp ratio and, correspondingly, a higher concentration of grape skin compounds (Singleton 1972, Doligez et al. 2013). Most grape flavonols, anthocyanins (Downey et al. 2006) and norisoprenoids (Gerós et al. 2012) are present in the berry skin. It has been shown, however, that 'relative skin mass (% of berry mass) was constant within (irrigation) treatments among the intermediate (berry diameter) categories' (Roby and Matthews 2004). Some skin compounds appear to be more concentrated in the skin of smaller berries (Roby et al. 2004); however, 'present results indicate that the source(s) of variation in berry size are more important in determining must composition and wine sensory properties, than berry size *per se*'. (Matthews and Kriedemann 2006). Most of the published work on berry diameter have focused on red grape cultivars and their phenolic substances (Roby et al. 2004, Barbagallo et al. 2011), and few data are available for white grape cultivars and aroma compounds (Šuklje et al. 2012). While red wine fermentation takes place in contact with berry skins, in white wine

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production skin contact time is limited, and therefore the contribution of skin compounds to wine composition may not be directly comparable.

Colorimetry is an approach to evaluate grape colour from diffuse light reflectance that is then converted into values that represent human visual perception according to the Commission Internationale de l'Eclairage (CIE) tristimulus values (CIELab). Assessment of berry colour according to the CIELab colour system has been widely used to evaluate the colour of different fruits, including grape (Carreño et al. 1995, Lancaster et al. 1997). The CIELab system describes the colour in a three-dimensional colour space with the L* (lightness), a* (red-to-green) and b* (blue-to-yellow) parameters. Berry colour is determined by the concentration of its skin pigments, which include carotenoids, chlorophylls and flavonoids. Many pigments (e.g. anthocyanins and carotenoids) are per se important determinants of grape composition. Furthermore, grape colour changes caused by pigment interaction with sunlight correlate with the accumulation of fruit compositional parameters (Skinkis et al. 2010). Thus, grape colour is a promising parameter for application in berry sorting operations. In the wine industry, CIELab measurements have been mostly used for red grapes in the assessment of anthocyanin concentration (Fernández-López et al. 1998) and composition (Liang et al. 2011). For white winegrape cultivars, the direct relationship between wine composition and berry colour has rarely been studied (Lehmen et al. 2011, Lafontaine and Freund 2013).

The a* value is the most variable of the CIELab coordinates under field conditions (Matthias Friedel, unpubl. data, 2011) and correlates well with the concentration of grape aroma compounds (Skinkis et al. 2010). It appears to be a well-suited colour coordinate to conduct berry sorting when targeting aroma compounds. The aim of this work was to investigate the relationship between berry colour (a* value) and/or berry diameter, and determinants of wine composition, including monoterpenes, norisoprenoids, phenolic compounds and organic acids in the white grape cultivar *Vitis vinifera* L. cv. Riesling.

Materials and methods

Grapes

Grapes of the cultivar *V. vinifera* L. cv. Riesling (clone Gm 198 grafted to rootstock 5C) were collected from an established vineyard of the Hochschule Geisenheim University, Germany (Geisenheimer Kläuserweg, approx. 49° 59′20″ N; 7° 55′56″E). The soil is deep, calcareous, with a predominance of clay and some marl. The vineyard has a steep slope and is south facing. Row orientation is north–south (N–S). About 10 kg of grapes were hand harvested at maturity on the 30 October 2013, approximately 70 days post-veraison. After harvesting, all berries were cut off the rachis with the pedicel base left on the berry. All broken or botrytised berries were discarded. Around 9000 berries were collected.

Berry diameter and density segregation

The berries were first sorted according to their diameter using four sieves with mesh diameter of 8, 10, 12.5 and 14 mm. Berries from the two smallest groups (<8 and 8–10 mm) were discarded as they represented only 5.4% of the population. The remaining three berry diameter groups, small diameter (10–12.5 mm), S1; medium diameter (12.5–14 mm), M1; large diameter (>14 mm), L1, representing 94.6% of the population were then sorted separately according to their density using a flotation method to achieve homogeneity in sugar concentration (Fournand et al. 2006, Kontoudakis et al. 2011). Six flotation solutions of different concentration were made by dissolving sodium chloride in distilled water (125, 135, 145, 155, 165 and 175 g/L). Difference in total soluble solids (TSS) between each density group was 1°Brix (Lafontaine and Freund 2013). One additional flotation solution (200 g/L) was made to indicate the upper limit of the range of berry TSS in the sample set. After flotation, berries were rinsed with distilled water to remove saline residues. One density group was chosen (135 g/L), and a subsample of 140 g of berries in triplicate was selected from each diameter group in order to compare berries differing in the diameter but homogenous in terms of TSS. The nine subsamples were stored at 4°C for winemaking on the following day.

Berry diameter and colour segregation

The M berries and the L berries of both the 155 and 165 g/L density class were each separately combined providing a total of 1041 M berries and 459 L berries, which were then segregated by colorimetry. Reflectance in the visible (VIS) part of the spectrum of the berry surface was measured from two opposite sides of each berry with a spectrophotometer (Minolta 3500d, Konica Tokyo, Japan), using a 3 mm aperture, the D65 illuminant and a 10-degree standard observer. The CIELab parameters lightness (L*), red/green (a*) and yellow/blue (b*) were calculated from the spectra. The colour-axis a* (red to green) was used to build two groups for each diameter class: high a* value and low a* value. Sample size was 140 g (100 M berries or 80 L berries). The colour groups of berries were built to obtain the same average and standard deviation value for both diameter groups. This was achieved by sorting the berries of each diameter class by a* value and calculating a running average a* value for 300 berries (100 berries per replicate, three replicates) of the M and 240 berries of the L berry group (80 berries per replicate, three replicates). An equal average with maximum possible difference of high and low a* value groups was selected for sample groups in both diameter classes. The berries of each sorted set were then assigned at random, successively from highest to lowest a* value, to three replicate sets, resulting in the following groups: MR [medium diameter (12.5–14 mm), high a* value], MG [medium diameter (12.5–14 mm), low a* value], LR [large diameter (>14 mm), high a* value], LG [large diameter (>14 mm), low a* value].

Winemaking procedure and fermentation

The samples were mechanically pressed on the next day (about 30 h after harvest) in a pressure controlled sample press at 100 kPa (Longarone 85, QS System, Norderstedt, Germany) for 10 min. Pressing was stopped briefly after 2 min to allow stirring of the must. Mean press yield of juice was 74% of fresh mass. The juice was collected in a beaker and instantly sulfited [140 µL sulfur dioxide (SO_2) as 5% v/v solution]. Juice (80 mL) was centrifuged (5430R, Eppendorf, Hamburg, Germany) for 7 min at 6000 *g* and 20°C. A sample (1 mL) of the juice was collected for standard juice analysis, and 70 mL of clear juice was transferred to a 100 mL brown bottle fitted with an air-lock to conduct the fermentation. Juice was inoculated with 25 mg/L of yeast (*Saccharomyces cerevisiae*, strain LW 317-28, Oenoferm, Erbslöh, Geisenheim, Germany). During fermentation at room temperature (20°C), mass and temperature of the fermenters were registered at least once a day. Fermentation temperature was 21–22°C. Fermentation was considered as completed after 17 days when the residual sugar of all samples was below 9 g/L. The fermented samples were centrifuged for 6 min at 6000 *g* and 20°C. A subsample of 50 mL of the supernatant was frozen

at −80°C for aroma analysis, 1.5 mL used directly for standard wine analysis while the remainder was frozen at −20°C for analysis of phenolic substances.

Juice analysis

A sample of 1.5 mL of juice or wine was centrifuged for 5 min at 1400 *g* (MiniSpin Plus, Eppendorf, Wesseling Berzdorf, Germany) and the supernatant measured with Fourier-transform infrared spectroscopy (FTIR; OenoFoss, FOSS, Hillerød, Denmark) providing results for organic acids, total titratable acidity (TA) (expressed as tartaric acid), pH and TSS in juice or residual sugars, TA, malic acid and alcohol in wine. The concentration of primary amino acids in juice was determined according to the NOPA procedure of Dukes and Butzke (1998). In brief, the amino acid groups were derivatised with *o*-phthaldialdehyde/N-acetyl-L-cysteine (OPA/NAC) reagent and absorbance at 335 nm was measured with a UV/VIS spectrometer (SPECORD 500, Analytik Jena, Jena, Germany) against a juice blank. Results were calculated as mg isoleucine equivalents from a standard curve.

Analysis of phenolic substances

Iron reactive phenolic substances were measured according to the Harbertson–Adams assay (Harbertson and Spayd 2006). In brief, wine samples were mixed with a triethanolamine/sodium dodecyl sulfate (TEA/SDS) buffer [containing 5% TEA (v/v) and 10% SDS (w/v) adjusted to pH 7.9] in a microcuvette. Background absorbance of the solution was read at 510 nm after 10 min, and again after the addition of 125 µL ferric chloride reagent (10 mmol/L $FeCl_3$ in 0.01 N HCl). All measurements were in duplicate and averaged. The concentration of iron reactive phenolic substances was calculated from a standard curve as catechin equivalents.

Aroma extraction and analysis

Aroma compounds were extracted using a protocol modified from Günata et al. (1985) and Kotseridis et al. (1998). Wine (40 mL) was diluted with 40 mL of deionised water (50%) and 8 µL of an internal standard (octan-3-ol, 50 µg/L and 2,6-dimethylhept-5-en-2-ol (DMH), 25 µg/L) was added and passed through SPE-cartridge (Strata SDB-L, 500 mg styrene-divenylbenzen polymer, Phenomenex, Torrance, CA, USA) previously activated using 5 mL of pentane/dichloromethane [2:1, (v/v)], 10 mL of MeOH, 10 mL of MeOH / H_2O [1:1, (v/v)] and 10 mL of water. The cartridges were flushed using 50 mL of water and dried for 30 min under vacuum under nitrogen flow (100 mL/min). Then, the free aroma compounds were eluted by pentane/dichloromethane [2:1 (v/v); 5 and 3 mL]. The extract was dried by adding anhydrous Na_2SO_4 and concentrated to 100 µL in a Vigreux column at 42°C. Extracts were stored at −18°C until analysis. The glycoside fraction was eluted using ethyl acetate; solvent was removed in a rotary evaporator (40°C; 138 kPa; 75 rpm) to dryness. The residue was dissolved in 7 mL of 0.2 mol/L citric acid (pH 2.5). Glycosides were hydrolysed exactly for 1 h at 100°C. Internal standard solution was added resulting in 50 µg/L DMH and 100 µg/L 3-octanol related to initial sample volume and liquid–liquid extraction was conducted, using pentane/dichloromethane (2:1, (v/v); 2, 1 and 1 mL). After drying with anhydrous Na_2SO_4, extracts were concentrated to 100 µL under gentle nitrogen flow (50 mL/min) and stored at −18°C until analysis.

Extracts were analysed using a Trace GC Ultra GC equipped with a PTV Injector and coupled to a ITQ 900 Ion Trap MS mass spectrometric detector (Thermo Fisher, Darmstadt, Germany). Gas chromatographic separation was carried out using a 30 m × 0.25 mm ID × 0.5 µm film thickness Agilent DB-Wax capillary column (Agilent Technologies, Santa Clara, CA, USA). An aliquot (1 µL) of the sample extract was injected in splitless mode (splitless time 90 s) at 220°C. The column oven was held at 40°C for 1 min during injection and then ramped to 60°C at 10°C/min, from 60 to 200°C at 3°C/min and from 200 to 230°C at 10°C/min, which then was held for 10 min. The carrier gas was helium with a constant flow of 1.4 mL/min and an average velocity of 27 cm/s. The interface and MS source temperature were set to 240 and 200°C, respectively, and MS data were acquired in electron impact mode with ionisation energy of 70 eV. Selected ion monitoring mode was used throughout each sample run with selected ions (Tables S1,S2) being used for the quantification of each aroma compound during post-run data analysis.

Statistical analysis

All data were expressed as the arithmetical average of the three replicates per treatment. The results were subjected to statistical analysis with the open source R 3.0.1 statistical computing environment (R Development Core Team 2006). One-way ANOVA was conducted for the first trial on the main factor berry diameter. Two-way ANOVA was conducted on the second trial with the two main factors, berry diameter and berry colour, and their interaction. Differences between treatment means were compared using the Tukey honestly significant difference test. Principal component analysis (PCA) was conducted using the MatLab PLS Toolbox (Eigenvector, Eagle Rock, CA, USA). Auto scaling was applied before calculating the model.

Results

Berry dimension, colour and density

About 9000 berries were sorted according to their diameter. Their distribution followed a standard Gaussian curve with the majority of the berries (49%) allocated to the M diameter group (12.5–14 mm diameter). The S diameter group (10–12.5 mm) and the L diameter group (14–16 mm) represented 31 and 14% of the berries, respectively.

The concentration of the salt solution showed a high correlation with the TSS of the juice (°Brix = 0.1408*x − 1.882, $R^2 = 0.88$). Within all berry diameter classes, TSS was highly heterogeneous and ranged from below 16 to above 21°Brix (Figure 1). An unexpectedly high proportion of berries (36%)

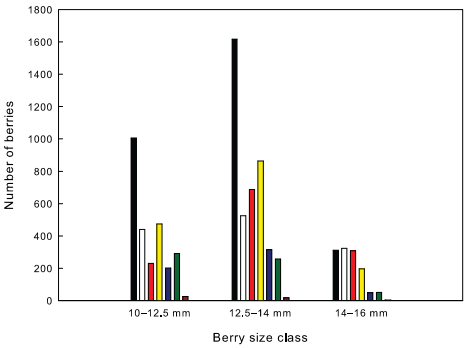

Figure 1. Distribution of berry ripeness, < 16 (■), 16–17 (□), 17–18 (■), 18–19 (■), 19–20 (■), 20–21 (■) and >21°Brix (■), in three berry diameter classes of *Vitis vinifera* L. cv. Riesling, 10–12.5, 12.5–14 and 14–16 mm.

Table 1. Composition of juices and wines from the first trial obtained from the three classes of berry diameter.

	Small diameter berries S1	Middle diameter berries M1	Large diameter berries L1	*P*_ANOVA	sig.
Juice					
Mean mass of a single berry (g)	0.98 ± 0.01 c	1.40 ± 0.01 b	1.77 ± 0.01 a	<0.001	***
TSS (°Brix)	17.37 ± 0.23	17.60 ± 0.00	17.22 ± 0.13	0.058	ns
TA (g/L)	10.27 ± 0.12 b	10.73 ± 0.29 ab	11.03 ± 0.12 a	0.008	**
Tartaric acid (g/L)	9.83 ± 0.25	9.70 ± 0.26	9.70 ± 0.17	0.733	ns
Malic acid (g/L)	2.10 ± 0.01 c	2.60 ± 0.17 b	2.97 ± 0.15 a	<0.001	***
pH	2.87 ± 0.01	2.84 ± 0.03	2.85 ± 0.01	0.116	ns
NOPA (mg/L)	96.67 ± 0.58	91.67 ± 2.08	99.67 ± 11.59	0.404	ns
Wine					
Ethanol (g/L)	87.76 ± 2.74	88.93 ± 1.62	86.00 ± 0.87	0.243	ns
Residual sugar (g/L)	5.37 ± 1.24	3.20 ± 3.93	2.90 ± 1.83	0.492	ns
TA (g/L)	9.63 ± 0.21 b	10.03 ± 0.11 a	10.23 ± 0.06 a	0.005	**
Malic acid (g/L)	2.23 ± 0.115 c	2.90 ± 0.01 b	3.20 ± 0.01 a	<0.001	***
pH	2.75 ± 0.04	2.73 ± 0.02	2.75 ± 0.01	0.626	ns
Volatile acidity (g/L)	0.26 ± 0.01	0.24 ± 0.01	0.24 ± 0.01	0.082	ns
Iron-reactive phenolic substances (mg/L)	340.14 ± 5.54	284.05 ± 61.19	272.86 ± 22.38	0.143	ns

Mean ± standard deviation ($n = 3$). Statistical difference between the treatments was assessed by a one-factorial ANOVA (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Different letters mark significant differences among groups as obtained by Tukey HSD test ($P < 0.05$). NOPA, nitrogen by *o*-phthaldialdehyde; ns, not significant; sig., significance; TA, titratable acidity; TDN, 1,1,6-trimethyl-1,2-dihydronaphthalene.

were unripe with TSS lower than 16°Brix. As a consequence, the distribution of the berries according to their density did not follow a normal distribution. The L diameter class presented the lowest proportion of unripe berries and the most homogeneous ripeness distribution. When fitting the data of each class, however, to a Gaussian equation { $y = a*\exp(-0.5*[(x-x0)/b]^2$}, the x0 parameter indicated the peak was located at 19°Brix for the S and M diameter groups whereas it was lower at 16°Brix for the L diameter group.

First trial: comparison of berries homogeneous in TSS but differing in diameter

Mean berry mass was significantly different ($P < 0.001$) among the S1 (0.99 ± 0.01 g), M1 (1.40 ± 0.01 g) and L1 (1.78 ± 0.01 g; Table 1) groups. For the three diameter groups TSS was similar ($P = 0.058$) at about17°Brix.

Though juices were homogeneous in TSS, the TA was 0.76 g/L lower in juice from small diameter berries S1 (10–12.5 mm) when compared with the juice from the large diameter berries L1 (14–16 mm) ($P = 0.008$, Table 1). A similar trend was shown for malic acid concentration ($P < 0.001$). No difference in pH ($P = 0.116$), tartaric acid ($P = 0.773$) and α-amino acid concentration (NOPA; $P = 0.404$) was observed among the groups. The fermentation rate was comparable for all berry diameter classes (Figure S1). At the end of the fermentation all wines had a residual sugar concentration lower than 9 g/L. There was no difference in sugar or ethanol concentration among treatments. Similar to juice analysis, TA in wines was 0.6 g/L lower for wines from the S1 berries compared with that of the L1 berries ($P = 0.005$), possibly related to a difference in wine malic acid concentration ($P < 0.001$). Sorting berries according to their diameter had no impact on the concentration of total iron reactive phenolic substances ($P = 0.143$, Table 1). Juice analysis results calculated as content per berry are shown in Table S3.

On average, 33% of total aroma compounds (198.44 ± 4.73 μg/L) in wines were present in free form and 66% as glycosides (390.56 ± 14.37 μg/L). Free aroma compounds were mostly represented by free monoterpenes (186.30 ± 4.60 μg/L, Table 2), whereas the concentration of free norisoprenoids was much lower (12.14 ± 0.13 μg/L). Hotrienol (52%) and α-terpineol (33%) were the most abundant glycosidically bound monoterpenes. The bound form of 1,1,6-trimethyl-1,2-dihydro-naphthalene (TDN) was present in high concentration, representing 77% of the bound norisoprenoids. It was not detected in wines in its free form; free norisoprenoids were mostly vitispirane (68%) and ß-damascenone (32%). Sorting berries by diameter had little impact on the concentration of monoterpenes or norisoprenoids of the resulting wines. Only the concentration of *cis*-linalool oxide was lower in wines from the S1 group compared with that of the M1 and L1 groups ($P = 0.013$). In contrast, berry diameter was negatively correlated to the concentration of free and bound α-terpineol and free linalool (Table S5). Compared with large berries, small berries showed about 10% higher concentration of total monoterpenes and an equal concentration of total norisoprenoids.

Second trial: comparing berries differing in diameter and colour but homogeneous in TSS

Berry mass was 1.37 ± 0.01 g for the M and 1.75 ± 0.04 g for the L diameter groups. No difference in TSS was observed among the four groups ($P = 0.077$, average 19.74°Brix). Colour distribution within both diameter classes was normal, and a* values measured over the whole berry population were within the range between −1.3 and +3.9. On average, a* values were higher for the M diameter group with 0.31 ± 0.73 compared with the L diameter group with 0.17 ± 0.59. The L* (30.15–29.81) and b* values (2.41–1.88) showed a similar trend. It is worth noting that while a* values were kept equal in low and high a* value groups, there was an interaction effect between berry diameter and b* value (blue-yellow): M diameter berries had a significantly higher b* value than L diameter berries with an average of 2.18 and 1.90, respectively (Table 3).

A two-factorial ANOVA on diameter and a* values showed that TA was associated with berry colour ($P = 0.029$, Table 4) but not with berry diameter ($P = 0.843$). Juice from low a* berries

Table 2. Concentration of monoterpenes and C_{13}-norisoprenoids in wines from the first trial obtained from the three classes of berry diameter.

	Concentration (µg/L)				
	Small diameter berries 10–12.5 mm	**Middle diameter berries 12.5–14 mm**	**Large diameter berries 14–16 mm**	***P*_ANOVA**	**sig.**
Free terpenoids					
Linalool	58.92 ± 4.62	53.8 ± 5.19	47.38 ± 4.81	0.072	ns
Nerol	nd	nd	nd		
Geraniol	11.99 ± 1.1	12.78 ± 1.04	12.71 ± 0.65	0.559	ns
Hotrienol	94.15 ± 9.7	102.39 ± 8.25	101.38 ± 14.41	0.635	ns
α-Terpineol	15.66 ± 1.51	12.62 ± 2.13	10.68 ± 2.52	0.069	ns
Nerol oxide	nd	nd	nd		
cis-Linalool oxide	6.15 ± 0.76 b	8.15 ± 0.45 a	7.63 ± 0.46 a	0.013	**
trans-Linalool oxide	0.84 ± 0.14	1.03 ± 0.25	1.38 ± 0.33	0.097	ns
Total free monoterpenes	187.71 ± 17.47	190.04 ± 14.18	181.16 ± 21.02	0.830	ns
TDN	nd	nd	nd		
β-Damascenone	4.01 ± 0.24	3.89 ± 0.63	3.86 ± 0.59	0.935	ns
Vitispirane	8.27 ± 0.06	8.19 ± 0.14	8.19 ± 0.15	0.662	ns
Total free norisoprenoids	12.28 ± 0.31	12.08 ± 0.49	12.05 ± 0.46	0.783	ns
Glycosidically bound terpenoids					
Linalool	2.11 ± 0.35	1.39 ± 0.46	1.56 ± 0.57	0.226	ns
Nerol	nd	nd	nd		
Geraniol	4.25 ± 0.26	4.03 ± 0.14	4 ± 0.09	0.251	ns
Hotrienol	59.48 ± 10.75	59.08 ± 11.22	59.36 ± 3.18	0.999	ns
α-Terpineol	48.79 ± 5.37 a	34.91 ± 2.85 b	28.31 ± 0.63 c	0.001	**
Nerol oxide	3.06 ± 1.62	4.14 ± 1.17	3.98 ± 1.22	0.601	ns
cis-Linalool oxide	7.39 ± 0.8	7.68 ± 1.41	6.34 ± 1.09	0.372	ns
trans-Linalool oxide	1.06 ± 0.12	1.08 ± 0.2	0.92 ± 0.05	0.322	ns
Total glycosidically bound monoterpenes	126.14 ± 11.7	112.32 ± 14.28	104.46 ± 6.19	0.134	ns
TDN	211.86 ± 123.66	216.8 ± 15.69	209.55 ± 21.67	0.992	ns
β-Damascenone	1.79 ± 0.29	1.69 ± 0.08	1.58 ± 0.03	0.408	ns
Vitispirane	60.53 ± 23.44	61.53 ± 3.6	63.27 ± 4.63	0.971	ns
Total glycosidically bound norisoprenoids	274.19 ± 147.02	280.17 ± 13.49	274.4 ± 26.06	0.996	ns

Mean ± standard deviation ($n = 3$). Statistical difference between the treatments was assessed with an ANOVA, post-hoc Tukey test (*, $P < 0.05$; **, $P < 0.01$). nd, not determined; ns, not significant; sig., significance; TDN, 1,1,6-trimethyl-1,2-dihydro-naphthalene.

Table 3. Mean L*, a* and b* values of the two berry groups of the second trial.

Berry class		**M§**	**L¶**
R†	L*	30.22 ± 1.5	30.08 ± 2.44
	a*	0.63 ± 0.21	0.63 ± 0.48
	b*	2.75 ± 0.92	2.49 ± 1.10
G‡	L*	29.73 ± 1.87	29.67 ± 1.97
	a*	−0.28 ± 0.17	−0.28 ± 0.23
	b*	1.61 ± 0.83	1.31 ± 0.78

Mean ± standard deviation. †Berry groups with high a* value. ‡Berry groups with low a* value. §Berry groups with medium diameter (12.5–14 mm). ¶Berry group with large diameter (14–16 mm). L*, lightness in the CIELab system; a*, red-to-green in the CIELab system, b*, blue-to-green in the CIELab system.

had higher TA and malic acid concentration than that of juice from high a* berries. Irrespective of berry diameter, juice from berries with lower a* values had a significantly higher concentration of α-amino acids (NOPA; $P = 0.019$). Juice from L berries had a significantly lower tartaric acid concentration and higher malic acid concentration than that of M diameter berries.

The fermentation pattern was similar between the treatments (Figure S1). As a result, the concentration of residual sugar and ethanol in wines was similar. Wine from L berries had a lower TA and a lower pH than wine from M berries, but differences were rather small. Interactions between diameter and colour were significant for these two parameters. Apart from a higher TA, wines from greener berries showed little difference compared with wines from more reddish berries. Neither differences in berry diameter nor colour had an impact on the concentration of iron-reactive phenolic substances in wines ($P = 0.573$, Table 4). Juice analysis results calculated as content per berry are shown in Table S4. A full correlation table between berry mass and colour parameters and wine chemical parameters is given in Table S6.

Aroma compounds in wines appeared to be more dependent on the colour differences than diameter differences (Table 5 and Figure 2). The concentration of total free and total bound monoterpenes was higher in wines from high a* berries as compared with that of wines from low a* berries. The same trend was observed for total free C_{13}-norisoprenoids. This was reflected in the principal component analysis, in which not a single aroma compound was associated with low a* values. Berry diameter also had a significant effect on wine aroma compounds. Wines produced from M berries had a higher concentration of total free monoterpenes and total glycosylated C_{13}-norisoprenoids compared with that of wines from the L berries. Wines produced from the M berries, however, showed a lower concentration of total bound monoterpenes (Table 5 and Figure 2). The concentration of individual aroma compounds was significantly influenced by berry diameter. Wine from M

Table 4. Composition of juices and wines in the second trial obtained from the two groups of berries sorted according to berry diameter and a* values.

Parameters	Medium diameter berries		Large diameter berries		Diameter		Colour		Diameter*Colour	
	Low a*	High a*	Low a*	High a*	*P*_ANOVA	sig.	*P*_ANOVA	sig.	*P*_ANOVA	sig.
Juice										
Mean mass of a single berry (g)	1.36 ± 0.01	1.38 ± 0.01	1.74 ± 0.05	1.75 ± 0.02	<0.001	***	0.502		0.886	
TSS (°Brix)	19.74 ± 0.13	19.89 ± 0.01	19.66 ± 0.01	19.66 ± 0.23	0.052		0.947		0.551	
TA (g/L)	10.43 ± 0.15	10.07 ± 0.21	10.27 ± 0.06	10.20 ± 0.10	0.843		0.029	*	0.104	
Tartaric acid (g/L)	9.51 ± 0.10	9.33 ± 0.21	9.13 ± 0.06	9.23 ± 0.25	0.048	*	0.747		0.219	
Malic acid (g/L)	2.81 ± 0.01	2.60 ± 0.20	3.03 ± 0.06	2.73 ± 0.06	0.028	*	0.007	**	0.488	
pH	2.87 ± 0.01	2.89 ± 0.02	2.89 ± 0.01	2.86 ± 0.01	0.540		0.837		0.013	*
NOPA (mg/L)	129.33 ± 2.08	122.33 ± 2.89	134.33 ± 8.02	122.33 ± 7.09	0.465		0.019	*	0.465	
Wine										
Ethanol (g/L)	101.80 ± 2.01	100.87 ± 0.45	100.23 ± 0.46	98.67 ± 2.57	0.085		0.229		0.750	
Residual sugar (g/L)	7.13 ± 4.31	3.40 ± 2.43	3.33 ± 4.91	5.73 ± 0.85	0.727		0.751		0.169	
TA (g/L)	10.13 ± 0.06	9.77 ± 0.11	9.87 ± 0.06	9.80 ± 0.10	0.048	*	0.003	**	0.017	*
Malic acid (g/L)	3.17 ± 0.11	2.96 ± 0.11	3.67 ± 0.05	3.07 ± 0.15	0.475		0.055		0.475	
pH	2.75 ± 0.01	2.79 ± 0.02	2.80 ± 0.00	2.79 ± 0.01	0.021	*	0.110		0.036	*
Volatile acidity (g/L)	0.25 ± 0.03	0.25 ± 0.01	0.24 ± 0.00	0.27 ± 0.01	0.512		0.207		0.207	
Iron-reactive phenolic substances (mg/L)	290.18 ± 57.89	345.08 ± 7.07	302.83 ± 8.13	312.20 ± 9.04	0.573		0.099		0.222	

Mean ± standard deviation ($n = 3$). Statistical difference between the treatments was assessed with a two-factorial ANOVA with factors colour and diameter (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). a*, red-to-green in the CIELAB system; NOPA, nitrogen by *o*-phthaldialdehyde; sig., significance; TA, titratable acidity.

Table 5. Concentration of free and glycosidically bound monoterpenes and C13-norisoprenoids in wines in the second trial.

	Concentration (µg/L)									
	Medium diameter berries		Large diameter berries		Diameter		Colour		Diameter*Colour	
	Low a*	High a*	Low a*	High a*	*P*_ANOVA	sig.	*P*_ANOVA	sig.	*P*_ANOVA	sig.
Free terpenoids										
Linalool	44 ± 1	61.5 ± 8	40 ± 1	58 ± 0	0.145		<0.001	***	0.901	
Geraniol	11 ± 1	13 ± 0	11 ± 1	12 ± 2	0.327		0.014	*	0.232	
Hotrienol	98 ± 3	124 ± 5	84 ± 2	117 ± 9	0.011	*	<0.001	***	0.323	
α-Terpineol	9 ± 0	15 ± 3	7 ± 1	15 ± 1	0.578		<0.001	***	0.451	
Nerol oxide	nd	nd	nd	nd						
cis-Linalool oxide	11 ± 1	13 ± 1	10 ± 0	12 ± 2	0.274		0.019	*	0.678	
trans-Linalool oxide	1 ± 0	1 ± 0	nd	1 ± 0						
Total free monoterpenes	173 ± 3	227 ± 15	153 ± 3	214 ± 9	0.016	*	<0.001	***	0.486	
TDN	nd	nd	nd	nd						
β-Damascenone	3.7 ± 0.1	3.5 ± 0.3	3.2 ± 0.1	3.9 ± 0.1	0.624		0.015	*	<0.001	***
Vitispirane	8.1 ± 0.1	8.3 ± 0.1	8.0 ± 0.0	8.2 ± 0.1	0.097		0.003	**	0.278	
Total free norisoprenoids	11.8 ± 0.1	11.7 ± 0.4	11.2 ± 0.1	12.2 ± 0.1	0.325		0.004	**	0.001	***
Glycosidically bound terpenoids										
Linalool	1 ± 0	2 ± 0	2 ± 1	4 ± 0	<0.001	***	<0.001	***	0.167	
Geraniol	4 ± 0	4 ± 0	4 ± 0	4 ± 0	0.015	*	0.004	**	0.003	**
Hotrienol	47 ± 5	58 ± 3	60 ± 6	88 ± 11	<0.001	***	0.001	**	0.068	
α-Terpineol	26 ± 3	38 ± 2	28 ± 7	55 ± 3	0.005	**	<0.001	***	0.010	**
Nerol oxide	3 ± 1	3 ± 0	2 ± 1	4 ± 1	0.979		0.019	*	0.168	
cis-Linalool oxide	6 ± 1	6 ± 1	4 ± 1	8 ± 2	0.843		0.012	*	0.037	*
trans-Linalool oxide	1 ± 0	1 ± 0	1 ± 0	1 ± 0	0.947		0.141		0.854	
Total glycosidically bound monoterpenes	87 ± 0	111 ± 5	100 ± 15	163 ± 16	0.002	**	<0.001	***	0.024	*
TDN	142.2 ± 7.1	161.5 ± 28.5	96.7 ± 25.0	124.2 ± 42.3	0.037	*	0.194		0.809	
β-Damascenone	1.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.6 ± 0.1	0.147		0.082		0.004	**
Vitispirane	56.6 ± 4.6	65.2 ± 10.2	47.1 ± 6.1	52.8 ± 11.0	0.055		0.179		0.773	
Total glycosidically bound norisoprenoids	200.2 ± 11.7	228.1 ± 38.8	145.1 ± 31.1	178.7 ± 53.1	0.039	*	0.187		0.897	

Mean ± standard deviation ($n = 3$). Statistical difference between the treatments was assessed with a two-factorial ANOVA with factors colour and diameter (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). a*, red-to-green colour in the CIELab system; nd, not determined; sig., significance; TDN, 1,1,6-trimethyl-1,2-dihydronaphthalene.

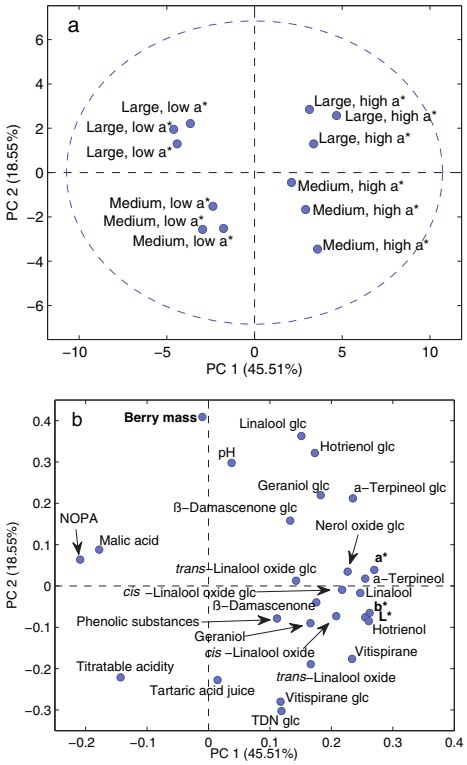

Figure 2. (a) Scores- and (b) loadings-plot of a principal component analysis conducted on the analytical, berry diameter and colour data from trial 2 on *Vitis vinifera* L. cv. Riesling grapes. glc, glycosylated compound.

berries had a higher concentration of free hotrienol and glycosylated TDN, and a lower concentration of bound linalool, geraniol, hotrienol and α-terpineol (Table 5). While the total terpenoid concentration of the wines was not affected by berry diameter ($P = 0.092$, data not shown), wines from M berries showed higher concentations of total norisoprenoids ($P = 0.039$).

Discussion

Berry diameter showed a normal distribution in our experiment, which is in accordance with other published data on Thompson Seedless (Kasimatis et al. 1975), Syrah (Barbagallo et al. 2011) and Sauvignon Blanc (Šuklje et al. 2012). Although 94% of the berries belonged to the L, M and S diameter classes, other cultivars and vintages may show a larger variance of berry diameter. Thus, studies spanning a larger range of berry diameter should be considered in future research. Sorting according to density showed a high variability of TSS, similar to other published results (Singleton et al. 1966, Fournand et al. 2006, Torchio et al. 2010). As shown in other studies (Fournand et al. 2006), there is a linear relation between berry density and TSS. Smaller berries showed a higher mean sugar concentration, just as found by other authors (Roby et al. 2004, Šuklje et al. 2012). Although fermented on a small scale, press yield of juice, fermentation rate and fermentation temperature were similar to commercial Riesling winemaking and did not show significant differences between sample groups in any of the trials. Microvinification is a common practice in viticultural and oenological research (Romano et al. 2003), albeit mostly conducted in somewhat larger volumes than 70 mL used in our study. As acids, monoterpenes and norisoprenoids are primary compounds, originating from the grape, comparable results can be expected in fermentations of larger scale. Shifts in the terpenoid profile and the ratio of glycosylated/free aroma compounds may, however, occur in larger fermentation volumes.

Berry diameter–related effects on grape composition

It has been shown that several environmental and genetic factors may influence berry diameter, among these are drought stress, nutrient availability, light interception and temperature at various developmental stages [reviewed by Dai et al. (2011)], in addition to seed number (Ribéreau-Gayon et al. 2006). A large part of berry diameter variation may be attributed to natural variability within a single bunch (Tarter and Keuter 2005), a single vine and between individual vines. In our study, we have investigated the naturally occurring variability of berry diameter, colour and composition as is represented in a commercial vineyard. It has often been assumed that skin compounds will be more concentrated in wines from smaller berries because of a higher skin to juice ratio (Singleton 1972, Weaver 1976, Doligez et al. 2013). It has been shown, however, that skin mass to flesh mass ratio remains relatively constant over intermediate berry diameter classes (Roby and Matthews 2004, Walker et al. 2005, Matthews and Nuzzo 2007). A fact that has remained undiscussed in these studies is that the berry surface area (through which a berry interacts with its environment) to total berry mass ratio increases with decreasing berry diameter. As a consequence, small berries heat up faster when exposed to radiation, but also cool down faster through forced convection, while larger berries reach a higher maximum temperature (Smart and Sinclair 1976). An increased surface to volume-ratio may also increase the loss of berry volatiles and increased respiration of berry compounds. Furthermore, under equal light exposure a larger amount of radiation per unit of berry mass is intercepted by a small berry compared with that of a large berry. Thus, radiation-dependent processes are accelerated in small berries. This is reflected by the fact that we observed an interaction between berry diameter and berry colour: Larger berries are greener than smaller ones. Malic acid concentration in juice and wine from smaller berries was lower than that in juice from large berries in both trials, although malate concentration at harvest is higher in berry skins than in berry flesh (Iland and Coombe 1988). While it is possible that smaller berries inherently have a lower malic acid concentration, 'levels of malate in harvested fruit may be largely determined by the rate of degradation during ripening' (Sweetman et al. 2009). Because of their larger surface to volume ratio, small berries may show a faster malic acid respiration during maturation as compared with that of large berries (Sweetman et al. 2009). This might explain the diameter-related effects on malic acid concentration in our study.

Tartaric acid concentration is much higher in the flesh than in the skin of berries. A decrease in tartaric acid concentration during ripening is believed to result from a dilution effect in the growing berry as the content on a per berry basis remains constant (reviewed by Terrier and Romieu 2001). This is a possible explanation for the berry diameter effect on tartaric acid concentration found in the second trial. In the first trial,

however, no such effect was observed. The tartrate/malate ratio was higher in juice from smaller berries in both trials. The difference in tartaric and malic acid concentration was rather small especially between M and L groups of both trials, and the difference found in TA was not consistent. The latter is in accordance with data published on the berry size–quality relationship of red cultivars (Walker et al. 2005, Barbagallo et al. 2011). The concentration of NOPA in juice and phenolic substances in wine was not influenced by berry diameter in either of the trials.

Effect of berry diameter on grape aroma composition

The analysis of aromatic compounds in trial 1 revealed few significant differences among berry diameter groups. This may be attributed to the rather large variance in the S1 group, of which one sample displayed unexpectedly low values, in particular for norisoprenoids. As small berries generally appeared to be less green and a lower a* value is associated with a higher concentration of aroma compounds (Skinkis et al. 2010), a stronger effect of berry diameter on aroma compound concentration was expected in this trial.

In trial 2, variation of berry colour was no longer random, which was the case in trial 1. Results of this trial revealed that the concentration of terpenoids in wine remained unaffected by berry diameter, while that of norisoprenoids was strongly elevated in smaller berries. This may be explained by the fact that the majority of carotenoids and norisoprenoids are located in the berry skin (Gerós et al. 2012), while the concentration of monoterpenes is equal or even higher in berry mesocarp compared with that in berry skin (Park et al. 1991). Hence, the carry-over of skin compounds into wine may be increased in wines from smaller berries. The relative proportion of free monoterpenes was elevated in M as compared with that in L berries. Smaller berries may show an intrinsic lower rate of terpenoid glycosylation or a higher rate of breakdown of terpene-glycosides, which is underlined by the strong correlation of berry mass with the proportion of free terpenes ($R^2 = 0.792$). In addition, the pH value of wines from M berries was lower than the pH value of wines from L berries, potentially leading to an increased hydrolysis of terpene-glycosides. Sorting by berry diameter and TSS produced samples that showed large variance between replicates, in particular for norisoprenoids. This may have contributed to a lack of significant findings in trial 1. Therefore, creating samples of equal colour should be considered in future research on berry diameter correlations with grape and volatile compounds in wine.

Effect of berry colour on grape and wine composition

Light interception of berry tissues strongly influences the concentration of grape skin pigments such as chlorophylls (Downey et al. 2004), carotenoids (Bureau et al. 1998, 2000), flavonols (Price et al. 1995) and anthocyanins (Dokoozlian and Kliewer 1996), and hence berry colour. While factors such as nutrient availability may also play a role in berry pigment accumulation (Linsenmeier and Löhnertz 2007), the influence of such factors was excluded by sampling bunches randomly from a homogeneous vineyard. We therefore assume that in our experiment light interception by the berries was a major factor behind changes in berry colour. Unpublished data from the 2011 growing season showed that complete shading of Riesling bunches reduced the variation (as measured by standard deviation) of the a* value of a bunch by 50%, whereas the standard deviation of the b* value decreased by only 10%, and L* value remained unchanged (Matthias Friedel, unpubl. data, 2011). These data highlight that the green–red axis of berry colour (a* value) is particularly affected by radiation. This may be explained by post-veraison bleaching of berry chlorophylls by sunlight (Downey et al. 2003).

Malic acid concentration in juice from less greenish berries was lower than that in juice from more greenish berries. Malic acid concentration is reduced by increasing light intensity after veraison (DeBolt et al. 2008), paralleled by the decrease in chlorophyll concentration (increase in a* value). An elevated concentration of malic acid may also explain the lower TA found in juice from berries with less greenish colour, which is in accordance with the results of Skinkis et al. (2010). Juice from more greenish berries showed a higher concentration of NOPA. Other studies (Kliewer and Ough 1970, Schultz et al. 1998, Gregan et al. 2012) showed that (UV) light exposure decreases the concentration of amino acids in grapes. The latter study indicated that the decrease in amino acids paralleled the accumulation of UV-screening pigments, such as flavonols, and thus changes in berry colour.

Our results show that differences in berry colour had a much larger influence on wine terpenoid concentration than berry diameter. Previous work showed that a strong positive relationship exists between the a* values and the glycosidically bound aroma precursors in berries measured by the glycosyl glucose method (Lafontaine and Freund 2013). Using an artificial shading approach, Skinkis et al. (2010) have shown that this relationship is caused by sunlight exposure in cool–moderate climates.

Sunlight is known to promote the accumulation of carotenoids (Mendes-Pinto 2009), leading to an increasingly yellow colour of fruit. Higher temperature and more sunlight have also been shown to increase degradation of carotenoids (Bureau et al. 1998), promoting the formation of norisoprenoids (Lee et al. 2007). Although b* value (yellow colour) was elevated in berries with elevated a* value, only a small colour effect on the concentration of free C_{13}-norisoprenoids was observed in our study, whereas glycosylated norisoprenoid concentration remained unaffected. It needs to be stated, however, that b* values correlated better with norisoprenoid concentration than a* values (Table S6). Hence, sorting by b* value may lead to a more pronounced difference in C_{13}-norisoprenoid concentration. It may be concluded that sorting berries by a* value leads to a pronounced difference in total terpenoid concentration, whereas norisoprenoid concentration is influenced more by berry diameter or b* value.

Consequences for practical grape sorting and winemaking operations

Winemakers have long sought to reduce the heterogeneity of grapes in order to maximise wine quality. Targeted berry sorting will greatly facilitate this task by reducing the variability of one or more of the physical/optical parameters, thus increasing wine quality (Keller 2010), or by removing undesired berry groups altogether. Berry sorting may prove helpful especially in premium winemaking, enabling winemakers to select grape material according to desired specifications. We have shown that, even in a homogeneous, well-watered, N–S oriented vineyard, grape composition is highly heterogeneous. Two potential parameters for berry sorting have been investigated in this study: berry colour (a* value) and berry diameter. Both parameters have been shown to be correlated, i.e. smaller berries are more yellow/red and less green/blue than larger ones. Therefore, either berry diameter or colour may be used in one-factorial berry sorting, and still reduce some variance of the second factor.

While the effect of berry diameter can be expected to be consistent in different climates, as they originate from the skin to juice and surface to volume ratio, berry sorting by colour may show different results under different climatic conditions. For example, berry a* value is strongly influenced by grape sun exposure and thus correlates with the concentration of berry terpenoids in cool climates. In hot climates, however, the concentration of terpenoids may decrease in exposed berries (Scafidi et al. 2013). Hence, it is possible that the opposite effects of berry sorting by colour will occur in hot climates, i.e. that greener berries are more aromatic than more reddish berries. More studies are therefore required to investigate berry sorting effects under different climatic conditions. Furthermore, the full spectrum of berry colour variation needs to be covered in future work. An automated berry colour measurement would facilitate that task.

Conclusion

This work underlines the complex nature of berry sorting operations: Sorting for one berry attribute will have intrinsic effects on other berry attributes. Under central European conditions, wines from smaller berries had a higher concentration of norisoprenoids and a higher tartrate/malate ratio. Small berries also tend to show a higher TSS. Berry a* value is positively correlated to free and glycosylated monoterpenes as well as to free norisoprenoids, and negatively to malic acid, TA and NOPA, even when TSS of the samples is similar. The data presented here may assist winemakers in their task to customise berry sorting operations to their specific needs.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site: http://onlinelibrary.wiley.com/doi/10.1111/ajgw.12210/abstract

Figure S1. Fermentation curves of all treatments, expressed as average mass loss. Error bars indicate the standard deviation. (a) First trial, berry diameter sorting: S1, small berries, 10–12.5 mm (●); M1, medium diameter berries, 12.5–14 mm (○); L1, large berries, > 14 mm (▼). (b) second trial, berry diameter and colour sorting: MR, medium diameter berries, 12.5–14 mm, high a* value (○); MG, (medium diameter berries, 12.5–14 mm, low a* value(△); LR, large diameter berries, > 14 mm, high a* value(●); LG, large diameter berries, > 14 mm, low a* value (▼).

Figure S2. (a) Scores – and (b) loadings-plot of a principal component analysis conducted on the analytical and berry diameter data from trial 1. glc, glycosylated compound.

Table S1. Applied standards in analysis of bound and free terpenes and C_{13}norisoprenoids, purity and suppliers.

Table S2. Details of the calibration used for the quantification of monoterpenes and norisoprenoids.

Table S3. Standard analysis in juice and wines obtained from the different diameter groups.

Table S4. Standard analysis in juice and wine obtained from the different groups sorted by berry diameter and a* values.

Table S5. Correlation analysis of berry mass and wine chemical parameters, trial 1.

Table S6. Correlation analysis of berry mass and berry colour parameters and wine chemical parameters, trial 2.

Chapter 6

General Discussion

Microclimatic effects on white grape quality have been underestimated

Thus far, only limited efforts have been dedicated to the research of microclimatic effects on the quality of white grapes. This lack of knowledge has led to an underestimation of the effects of microclimate manipulation on the chemical and sensory properties of the resulting wines. Knowledge about the effects of microclimate manipulation will however gain relevance to producers in order to maintain the regional typicality of wines in the context of changing climate conditions (Keller, 2010). The attention of German winegrowers focused on this topic in the aftermath of the 2015 growing season, when the effects of an untypically hot growing season were aggravated by the excessive application of leaf removal. Leaf removal had been recommended by consultants despite hot weather conditions throughout the growing season in several viticultural regions. The consequences thus far documented include the requirement for higher doses of bentonite for protein stabilization due to an accumulation of (heat shock) proteins. At the same time, the concentration of amino acids in white grape juices was quite low under the hot conditions of this growing season. This required winemakers to increase the amount of supplemental yeast available nitrogen added to the juice to ensure successful and stable fermentation. Light exposure in combination with high temperatures and drought stress further led to the development of a bitter or adstringent taste in wines caused by the accumulation of phenolic compounds, which required additional fining to meet market demands. This was often accompanied by a loss of fruitiness in typically light and fruity wines. As the frequency of such hot growing seasons is expected to increase due to climate change (IPCC, 2014), it is necessary to increase the awareness of both wine growers and consultants for the potential risks of excessive light exposure in such scenarios. The problem of untimely microclimate manipulation is however not only caused by a lack of awareness from the consultant side, but also by the growers' opinion that once the expensive leaf removal machinery is acquired, it needs to be used frequently to bring down fixed costs and thus "pay off" over time.

Investigating the effects of row orientation as a model for microclimatic effects

A drawback of using leaf removal as a tool to investigate the effects of microclimate manipulation is that the vines loose a part of their carbon supply source, which does then influence grape metabolism (Poni et al., 2006). Some researchers have thus resorted to sampling grapes grown under different levels of canopy shade (e.g. Reynolds and Wardle, 1989b). However, the reproducibility of light conditions around a grape bunch during the course of a day or even an entire season is poor when leaves are present around the bunch. Further, constant, homogenous shading of bunches only occurs in very dense canopies. We have thus tried to overcome this problem by using an artificial shading treatment which allows for the sampling of several bunches under homogenous conditions (Friedel et al., 2016a). Alternatively, large, homogenous grape samples grown under different microclimatic conditions in close spatial proximity can be obtained when bunches or berries from different sides of canopies cultivated in different row orientations are sampled.

Vines planted in different row orientations have different microclimates on the respective side of their canopy, as the canopy acts as a natural wall, blocking light penetration to the other side. Differences of assimilate supply do not occur between canopy sides within a given row orientation, as lateral and primary leaves of a given shoot are distributed roughly equal between canopy sides. Accordingly, no differences in sugar concentration are observed between these canopy sides. In an experimental vineyard of Geisenheim University planted in 2007 with Riesling (clone 239 on rootstock SO4), we have conducted four years of experimentation on the row orientations N-S, E-W, and NE-SW. The experiments consisted of the description of canopy and fruit temperatures using thermal imaging and thermocouples, and investigating possible differences between canopy sides of amino acid, organic acid, mineral nutrient and phenolic composition of the grapes. The studies were conducted in the years 2010-2013. Measurements with temperature sensors inside the canopy revealed the effects of row

orientation on canopy temperature. Figure 1 shows that different temperature regimes arise from different row orientations. The most notable difference among the orientations is the pronounced temperature maximum at around 16:00 MST in N-S oriented rows. However, although several simulation studies showed that N-S oriented rows have a higher light interception than all other orientations at most latitudes and under most conditions (Riou et al., 1989; Smart, 1973), mean day temperature differences were not significant at only 0,1 °C among the three row orientations. The extent of grape temperature heterogeneity on the respective canopy sides during the course of a day

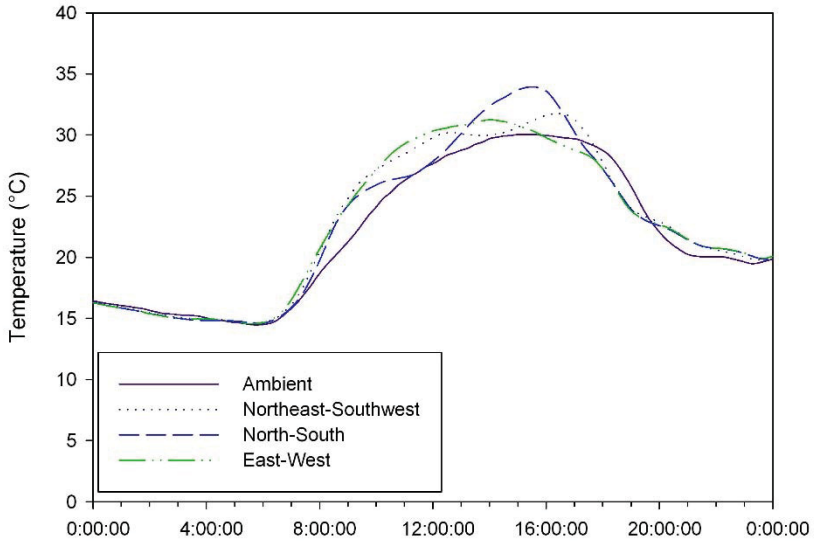

Figure 1: Diurnal temperature development inside the canopy of row planted in N-S, E-W and NE-SW orientation as compared to ambient temperature. Recorded 03.09.2011 in Rüdesheim (49° 59' 20'' N).

(figure 2) was measured by infrared thermography. Although the day on which the measurements were conducted was only a warm late-summer day (17.08.2011), the maximum berry temperature, measured at 15:15 on the western side of the N-S row orientation, reached 43 °C, which was about 15 °C warmer than the temperature of the eastern side of the same canopy, and roughly 10 °C warmer than a bunch on the same canopy side shaded by one leaf layer. Accordingly, we have documented the highest sunburn incidence during a major sunburn event in 2012 on the W side of N-S oriented rows. 25.7 % of all bunches showed sunburn damages in September, compared to only 3.7 % in E-W oriented rows. Similar outcomes were demonstrated by a growers survey assessing sunburn damage following a heat wave in south-eastern Australia (Webb et al., 2010).

The largest differences of mean berry temperature between the respective canopy sides were measured in E-W oriented rows, which is not surprising as

the northern side of the canopy in this row orientation receives almost no direct radiation during the ripening phase. The large differences of light and temperature between the two sides of a canopy manifest themselves in differences of metabolite accumulation. Among the largest differences between canopy sides were the concentration of flavonols in the berry skin and the concentration of amino acids total acidity and malic acid in grape juice (table 2).

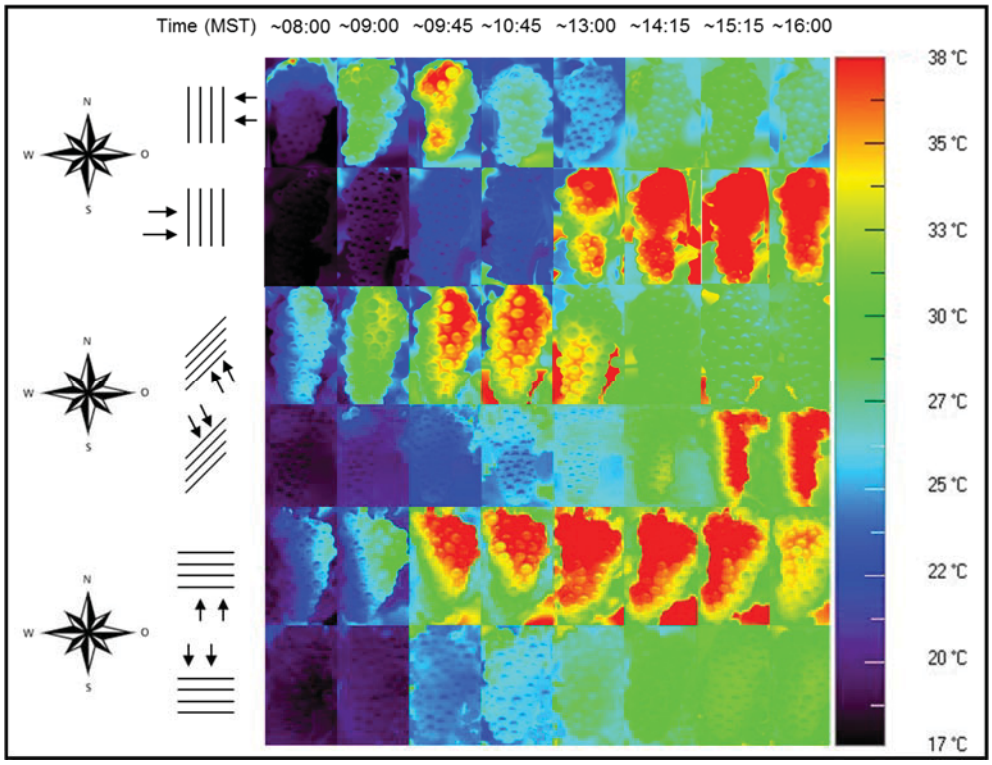

Figure 2: Berry temperature of different row orientations as measured by infrared thermography on *Vitis vinifera* L. cv. Riesling. Recorded 17.08.2011 in Rüdesheim (49° 59' 20'' N).

These results were all in accordance with the results obtained by targeted microclimate manipulation. However, the differences induced by row orientation were by far not as large when using artificial methods. This is not surprising, as naturally occurring differences in the illumination of the bunches under different row orientations can't be as large as with artificial microclimate manipulations. Under natural conditions, 9 % of the incoming radiation is transmitted through a single leaf layer (Smart, 1985), some light passes the canopy through gaps, and the diffuse radiation is equal on both canopy sides. Weiss et al. (2003) estimate the insolation distribution between the canopy side of an east-west canopy at a ratio of 4:1. In contrast, when using opaque boxes, the radiation reaching the bunches was less than 1 % of

the ambient radiation (Friedel et al., 2016a), explaining the rather large differences induced by artificial shading.

No canopy side effects were observed on the concentration of mineral nutrients in grape juice, but N, P and Ca concentration was elevated in more sun-exposed leaves in the years 2012 and 2013 compared to the leaves of less sun-exposed canopy sides. For example, N, P and Ca were 6, 16 and 12 % elevated, respectively, in south-exposed leaves of an E-W oriented canopy as compared to north-exposed leaves.

Table 2: Variability of some grape compounds under different row orientations. Measurements were taken on the cultivar Riesling (*Vitis vinifera* L.) in the years 2010-2013 (Phenolic compounds 2011-2013) in a vineyard in Rüdesheim (49° 59' 20'' N). Significant differences between canopy sides are marked in italic type.

Orientation	Northeast-Southwest		North-South		East-West	
Canopy side	Northwest	Southeast	East	West	North	South
Phenolic conmpounds (mg/g skin fresh wt)						
Flavanols	*0.17 ± 0.04*	*0.14 ± 0.04*	0.17 ± 0.02	0.16 ± 0.05	0.13 ± 0.03	0.12 ± 0.04
Hydroxycinnamic acids	0.57 ± 0.16	0.57 ± 0.12	*0.56 ± 0.08*	*0.47 ± 0.15*	0.41 ± 0.16	0.43 ± 0.15
Flavonols	*1.59 ± 0.51*	*2.21 ± 0.66*	1.58 ± 0.58	1.86 ± 0.53	*1.05 ± 0.28*	*2.12 ± 0.41*
N compounds (mg/L)						
Amino acid N	*236.0 ± 149.2*	*198.2 ± 106.7*	198.9 ± 124.0	202.0 ± 118.3	*220.5 ± 141.3*	*191.6 ± 110.9*
Ammonia	45.1 ± 10.8	45.2 ± 10.2	37.8 ± 12.8	38.3 ± 10.4	42.9 ± 10.2	42.8 ± 9.7
Acids (g/L)						
Malic acid	*3.81 ± 1.44*	*3.6 ± 1.28*	3.85 ± 1.24	3.67 ± 1.57	*3.72 ± 1.65*	*3.35 ± 1.35*
Total titratable acidity	*13.46 ± 1.75*	*12.91 ± 1.52*	*12.65 ± 1.47*	*13.38 ± 2.06*	*13.81 ± 1.82*	*13.1 ± 1.89*

The microclimatic, and thus metabolic, differences between canopy sides increase with the density of the canopy. All measures to reduce canopy compactness do thus not only improve phytosanitary conditions and grape quality, but also increase the homogeneity of the grapes. As heterogeneity of grape quality has been reported to be detrimental for wine quality, reducing canopy compactness will be beneficial for wine quality, especially when canopies have an orientation that deviates significantly from a N-S orientation. No significant differences were observed between most parameters when testing the effects of row orientation *per se* except for sugar concentration, which was significantly elevated in E-W compared to N-S or NE-SW oriented rows. This is in accordance with data modelled by Lebon et al. (1995), who have shown a slightly higher photosynthetic capacity of E-W oriented canopies in a VSP trellis system as compared to other orientations despite a lower light interception in this row orientation. In contrast, Intrieri et al. (1998) found no effect of row-orientation on total canopy assimilation of potted Sangiovese vines.

Timing is crucial for the application of microclimate manipulation techniques

Although there has already been some research on effects of timing of microclimate manipulation, the data available today do not allow for a comprehensive understanding of the matter.

In Chapters 3 and 4 we have shown that the timing of microclimate manipulation plays a crucial role for final grape quality. While Gregan et al. (2012) have published detailed data of the evolution of amino acids and the effect of UV light after leaf removal has been conducted shortly after bloom, the effect of different developmental stages for leaf removal was not investigated in that study. Apart from demonstrating that the main effect on amino acid concentration induced by leaf removal occurs post veraison and thus postponing leaf removal until after veraison will not help avoid problems with yeast nutrition (Friedel et al., 2015), we were able to reject the common hypothesis that the effect of leaf removal on amino acids is not a microclimate effect, but rather caused by the removal of the leaf as a source for amino acids (Petgen et al., 2004). This was achieved by introducing a complete shading treatment without manipulation of the microclimate. However, the mechanism governing the pronounced microclimatic influence on the accumulation of amino acids in the berry flesh and juice is as yet unknown.

In addition, we were able to demonstrate the effects of microclimate manipulation on various classes of phenolic substances during berry development. In accordance with already published data (Downey et al., 2003; Gregan et al., 2012; Price et al., 1995), we have shown that flavonol concentration in the berry skin increases drastically upon the interception of UV radiation, while the main period of synthesis is after veraison (Friedel et al., 2015; Friedel et al., 2016a). The HPLC methodology utilized in our studies allowed for the quantification of seven flavonol glycosides. For all of these glycosides, the kinetics of accumulation were a function of developmental stage and irradiation. As all flavonol glycosides seem to show a specific reaction upon illumination at any given developmental stage, the pattern of flavonol glycosides found in grapes may well be considered as a microclimatic library of its growing season. The application of discriminant analysis (iPLS-DA) to the flavonol data of Chapter 3 with their history of microclimate manipulation showed a validated classification accuracy of 100 %, and thus support this hypothesis.

Of the other classes of phenolic compounds, the concentration of hydroxycinnamic aids (HCAs) as well as flavanols was increased by radiation only during and shortly after flowering. No microclimatic effects on the concentration of these two classes of phenolic compounds were detected at veraison or later. Early leaf removal will therefore increase HCA and flavanol concentration in the grapes (Friedel et al., 2015), while a later defoliation will rather lead to an increase of flavonols (Friedel et al., 2016a). As the latter are being associated with bitter taste, while HCAs are associated with a positive mouthfeel, an early leaf removal is advisable from a phenolic composition point of view, providing the vineyard is sufficiently vigorous to allow for regrowth of leaves to cover the grapes by veraison.

Another crucial parameter for wine quality is the primary aromatic composition of grapes, which contributes a large part to the final wine flavor. A main focus of this thesis was the terpenoid fraction of the grape aroma (Friedel et al., 2016a), as this fraction defines the flavor of all "aromatic" grape cultivars, including Riesling. Controversial results have been published about the microclimatic influence on the synthesis of terpenoids (Bureau et al., 2000a; Bureau et al., 2000b; Scafidi et al., 2013). Our aim was to bridge this gap of knowledge by an extensive literature review and the application of quantitative RT-PCR on the recently published sequences of several important monoterpene synthases which were likely to be present in Riesling. To the best of our knowledge, this was the first study investigating the microclimatic influence on the gene expression of the terpenoid synthesis pathway in grapes. By reviewing literature published on the influence of microclimatic conditions on terpenoid synthesis, we have found a correlation between the results of the studies and the macroclimatic condition in which they were conducted: the studies in which monoterpene concentration was increased by shading or did not change were conducted in rather hot climates, the other studies showing a negative effect of irradiation on terpenoid accumulation in cool-moderate climates. The apparent positive contribution of light to terpenoid accumulation therefore appears to be out-weighed by an increased loss of terpenoids through additional solar heating and evaporation in hot climates.

The data shown in Chapter 4 add to this picture. In this study we have shown that the expression of monoterpene biosynthetic genes, including a monoterpene glycosyl transferase is indeed up-regulated by solar radiation and down-regulated by shading. This was reflected in a drastically increased monoterpene content of sun-exposed berries. We have further shown that the terpenoid content of the grapes at harvest was also affected by the timing of

microclimate manipulation: The terpenoid content of bunches shaded for three weeks post-veraison was drastically lowered compared to the control treatment at harvest, while the content of flavonols was equal in both treatments. This may be explained by the maximum expression of monoterpene biosynthetic genes, which occurs around veraison (Martin et al., 2012). In contrast, flavonol biosynthesis takes place mainly post veraison. To optimize grape aroma, growers therefore need to ensure light penetration shortly before and during veraison, when terpenoid biosynthetic gene expression is at a maximum. A recently published study on Sauvignon Blanc and Riesling observed similar effects of shading on total linalool levels. The authors have further shown that UV-light is of particular importance for the synthesis of linalool, and pointed out the importance of the early Methylerythritolphosphate (MEP) pathway genes for monoterpenol synthesis (Sasaki et al., 2016).

The optical properties of white grapes are influenced by the microclimate

Apart from parameters which are directly relevant for fruit quality, it has long been known that microclimatic effects can rapidly influence fruit color in white grapes (Kern and v. Babo, 1846). While red cultivars evidently undergo color change during veraison, the evolution of the optical properties of white grape is less known.

It appears that high irradiation leads to increased chlorophyll synthesis before veraison, while low-light conditions lead to decreased chlorophyll synthesis. A similar development has been reported for the xanthophyll cycle carotenoids in grape (Düring and Davtyan, 2002; Lee et al., 2007), as these pigments are closely associated with chlorophylls in the chloroplasts, where they play a role in light harvesting as well as in radical scavenging (Solovchenko, 2010). After veraison, it seems that chlorophyll of white grapes undergoes (photo-)degradation, unmasking the carotenoids (Mendes-Pinto, 2009) and changing berry color from green to yellow. The yellow color is apparently enhanced by the accumulation of flavonols in the epidermal vacuoles during ripening (Solovchenko, 2010). Enzymatic activity as well as oxidative reactions lead to the degradation of some carotenoids to aroma-active C_{13}-norisoprenoids, which might explain a generally decreasing concentration of carotenoids in the berry skins post veraison (Yuan and Qian, 2016). This process seems to be accelerated by radiation and high berry temperatures. Unpublished reflectance data recorded during the microclimate

studies introduced in chapters 3 and 4 have confirmed that the pigments of Riesling also behave according to that pattern. The variance on the green-red axis of the CIE L*a*b* color scale appeared to be more strongly influenced by light than the yellow-blue axis of the scale, indicating that light induced acceleration of the chlorophyll degradation precedes carotenoid breakdown after veraison. Similar data were obtained when recording VIS-spectra of berries on different canopy sides of three different row orientations. In figure 3, the color changes induced by radiation can be seen in the green peak reflection at around 550 nm which is higher in less light-exposed samples (N and N-E). In parallel, the red reflectance is decreased in these treatments, indicating a higher light absorption by chlorophyll pigments.

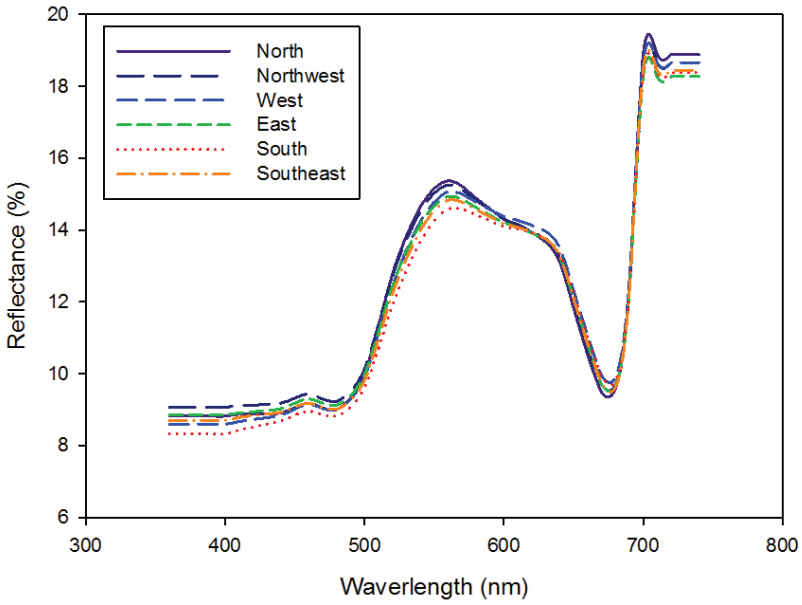

Figure 3: Mean reflectance spectra of 100 grape berries of different canopy sides of N-S, E-W and NE-SW row orientations. Grapes were taken from a Riesling (*Vitis vinifera* L.) vineyard in Rüdesheim (49° 59' 20'')

In this case, lower green reflectance and a higher red reflectance of sun-exposed samples (South, Southeast, West) as compared to samples from rather shaded canopy sides indicated a faster progress of chlorophyll degradation. A set of reflectance data taken from the samples that have been described in chapter 3 showed that shading or leaf removal influenced almost all optical properties of the grapes measured by a Multiplex fluorometer (Force-A, Paris, France).

Table 3: Confusion matrix of the validation set of an iPLS-DA on Riesling (*Vitis vinifera* L.) berry reflectance data taken at veraison with a Multiplex fluorometer (Force-A, Paris, France)

	Actual Class		
Predicted as	Shading	Control	Leaf removal
Shading	6	0	0
Control	0	6	0
Leaf removal	0	0	6

By applying discrimination analysis (iPLS-DA with 50/50 data split into training and validation sets) on these data, grapes could be easily classified to their respective microclimate treatments at veraison (table 3) and harvest (table 4). Thus, it is evident that grape optical properties and grape quality show co-variance patterns caused by microclimatic phenomena. As the classification of grape samples according to their respective microclimate was quite accurate by reflectance measurements, these co-variances might be technically exploitable, e.g. in fruit sorting operations.

Table 4: Confusion matrix of of the validation set of an iPLS-DA on Riesling (*Vitis vinifera* L.) berry reflectance data taken at harvest with a Multiplex fluorometer (Force-A, Paris, France)

	Actual Class				
Predicted as	Shade E-L 27	Shade E-L 34	Leaf removal E-L 27	Leaf removal E-L 34	Control
Shade E-L 27	4	0	0	0	0
Shade E-L 34	2	6	0	0	0
Leaf removal E-L 27	0	0	4	2	1
Leaf removal E-L 34	0	0	2	3	0
Control	0	0	0	1	5

Microclimatic effects can be exploited in fruit sorting

By definition, the quality testing of fresh fruit requires non-destructive measurements to eliminate faulty fruit prior to sale or further processing. In most cases, optical sensing methods in the VIS, NIR and MIR regions of the electromagnetic spectrum and often physical size grading represent the state of the art technology in fruit sorting. In almost all instances of non-destructive measurements, the quality related compound of interest is not homogenously distributed among all parts of the fruit (Alander et al., 2013). In the case of grapes, the berry skin, consisting mostly of structural carbohydrates, is not representative of the concentration of quality driving sugars, acids and other quality related compounds of the berry flesh. Thus, it is necessary to conduct

contact measurements in transmission or interaction mode to allow for a better signal of the compound in question when true measurements are desired. The process technology and the very nature of berry fruit, however, precludes the application of contact measurement as fruit vary in size and dozens of measurements have to be carried out simultaneously on a subsecond timescale to allow for a reasonable process speed. Thus, when conducting fruit quality control or sorting in berry fruit applications, reflectance measurements are applied, although they often do not allow for a direct measurement of the target compound, but rather have to exploit the covariance of berry optical and quality traits, which is not a direct correlation.

Grape berry sorting technologies have recently been introduced to the market (Hendrickson et al., 2016). The first generation of these systems have focused on the removal of material other than grape and botrytised fruit, but modern berry sorting technology can also conduct sorting by berry size and reflectance in various parts of the electromagnetic spectrum. This technology is already in use to sort ultra premium red grapes according to winemaker specification in the U.S. (Hendrickson et al., 2016). We have investigated the use of both UV/Vis spectrometry and VIS spectrometry combined with berry size sorting to test the feasibility of sorting grapes grown under different microclimatic conditions to reduce the heterogeneity of the harvested grapes (Friedel et al., 2016b). This is beneficial in large vineyards, where drastic differences of vine vigor can occur, but also in E-W oriented vineyards or the mountain side of steep slope vineyards. Sorting may also be applied when cultivars like Cabernet Sauvignon, Cabernet Franc or Sauvignon Blanc are processed. In these varieties, fruit grown in the shade may contain high amounts of methoxypyrazines, which add a capsicum-like taste to the wines, which is often perceived as being detrimental to quality. We had the prospect to select grapes with elevated amounts of TDN by simple VIS spectrometry and size sorting, but apart from gaining some effects from berry size sorting, were not able to obtain berry subgroups with different TDN concentration by color sorting. Possibly, sorting on the blue-yellow axis may yield berry subgroups with larger differences in TDN concentration, as carotenoids have large absorption maxima in the blue spectral range and often confer yellow color to fruit (Lichtenthaler and Buschmann, 2001). Nevertheless, sorting berries on the green-red axis of the CIE L*a*b* scale yielded large differences regarding the overall quality of the fruit: less green berries had a higher concentration of monoterpenes and a lower concentration of malic acid at equal levels of sugar and TDN (Friedel et al., 2016b). Of course, these results can only be the beginning of research on this topic. Berry sorting provides the winemaker with vast possibilities for the removal of undesired

fruit or the selection of grapes of defined quality in both both red and white grape processing.

Conclusions

In the course of this thesis, I have investigated the effects of row orientation, light exposure and shading on the quality and optical properties of white Riesling grapes. With our experiments on row orientation, we have demonstrated the immense impact that row orientation can have on berry temperature. Although row orientation is often dictated by site geometry, there are instances when growers can freely choose between different row orientations. In this case, advantages and drawbacks of the respective row orientations need to be known. We have shown that, caused by maximum berry temperatures, sunburn incidence is highest on the W side of N-S oriented rows. E-W oriented rows appear to have a slightly better ripening potential than N-S and NE-SW oriented rows and a lower sunburn incidence, but highly heterogenic fruit on the N and S side of the canopy regarding amino acid, phenol and organic acid composition, which may be detrimental for wine quality. All measures to reduce canopy density, such as leaf removal, hedging and suckering, can contribute to reduce the degree of heterogeneity.

The timing of such measures, however does itself influence fruit composition. We have shown that almost all compounds with relevance for final wine quality which we have investigated in our studies, react to changes in microclimate, most notably irradiation, as some stage of berry development. This was the case for amino acids, the concentration of which was decreased by light and increased by shade before as well as after veraison, various classes of phenolic compounds, malic acid and volatile compounds. Manipulating the microclimate at a given stage of development will thus give a specific imprint on the quality of the grapes at harvest.

The microclimate also influences the optical properties of a berry, mainly because of their effects on chlorophyll and carotenoid synthesis and degradation and phenol accumulation. These changes can be followed and discriminated by optical sensing and adequate data analysis. Both of the tested sensors, a VIS-spectrophotometer (Konica Minolta 3500d, Tokyo, Japan) and a Multiplex “polyphenolmeter” (Force-A, France), a portable fluorometer that has recently been introduced to viticultural research and practice, seem to be able to discriminate grapes grown under different microclimatic conditions, producing subsets of samples with different compositions of acids, phenolic and aroma compounds. Recent grape sorting technology allows to conduct berry sorting in different regions of the electromagnetic spectrum and thus sort grapes according to desired specification. More

research is needed to clarify which spectral region is best suited to conduct reproducible sorting for the respective target parameters.

The aims of my research efforts were to provide a deeper understanding of the consequences of microclimate manipulation at different stages of berry development. Using these novel insights, growers and consultants are enabled to develop novel strategies of targeted microclimate manipulation and utilize the microclimatic effects on the optical properties of grapes to produce wines of distinct sensory properties.

Chapter 7

Summary

Microclimatic effects on grape quality have been recognized for more than a century, when first experiments with leaf removal were published. The manipulation of microclimatic conditions in the canopy gained much attention in the 1960s and 70s, when the progress in rootstock breeding, the excessive use of mineral fertilizers and the establishment of vineyards on fertile soils led to hitherto unknown vine vigor. The consequences were increasingly dense canopies, which led to microclimatic conditions in the canopy which favored fungal infections of grapes and leaves, and ultimately to a decreased grape and wine quality. Practices like leaf removal became more widespread in the 1990s, when adequate machinery to conduct mechanical leaf removal became available. The possibility of mechanized leaf removal decoupled its application from the period of low workload after veraison, when it was traditionally conducted. Although a lot of research has been conducted regarding the effects of microclimate manipulation on the quality of red grapes, the vast majority of studies focused on the synthesis of anthocyanins and the timing of veraison.

The objective of this thesis was to provide a deeper understanding of the microclimatic influence on grape quality at different stages of berry development, with focus on the white grape cultivar Riesling (*Vitis vinifera* L.), using an optimized protocol for high-throughput FTIR measurements and other state of the art analysis to quantify compounds relevant for grape and wine quality.

Three different approaches to create variable microclimatic conditions were used: The removal of leaves in the bunch zone to maximize bunch exposure, the complete shading of bunches using opaque boxes and the utilization of the natural variance of the microclimatic conditions on different sides of the canopies of vines planted in different row orientations.

The effect of microclimate manipulation on different classes of phenolic substances was large in the case of flavonols, and rather small in the case of flavanols and hydroxycinnamic acids. The only effects of microclimatic conditions on flavanol and hydroxycinnamic acid concentration was observed when leaf removal or shading were conducted directly after berry set. The microclimatic effects on flavonols were rather large as was expected due to

their role in UV-protection: radiation at any developmental stage led to a rapid increase of flavonol concentration in the berry skin, whilst without radiation the synthesis of flavonols was inhibited completely. At all developmental stages, a specific pattern of flavonol glycosides is synthetized, depending on the expression of the respective flavonol glycosyl transferases during the respective developmental stages.

The picture with monoterpene synthesis was more complicated: Although they accumulate only at later stages of ripening, the most important period for their synthesis is around veraison, when the expression of monoterpene biosynthetic genes is at a maximum. Bunches shaded during the ripening phase had a barely detectable monoterpene concentration, and re-illumination during the last weeks of ripening did only increase their concentration by 2-fold, comprising only 20 % of the concentration of the control treatment. In the same time, flavonol concentration recovered rapidly to levels comparable to those of the control. Consequently, leaf removal at later ripening stages will mainly increase the concentration of flavonols in the berry skin, but have little effect on the concentration of aroma compounds.

The concentration of amino acids was strongly influenced by microclimatic conditions, with shading increasing and sun exposure decreasing the concentration of amino acids in the berries, but not in the leaves, which were tested in the row orientation experiments. Although these effects were demonstrated to happen already before veraison, the quantitatively more important changes occur when the bulk of amino acids begins to accumulate in the berries, i.e. post veraison.

Different row orientations lead to strongly different temperature regimes on the different sides of grapevine canopies, with maximum berry temperatures about 15 °C above ambient temperature being reached on the W side of N-S oriented canopies in the afternoon. This canopy side also showed the strongest sunburn incidence and the highest temperature inside the canopy. Infrared thermographic measurements also showed that shading by one leaf layer can decrease fruit temperature by more than 10 °C. Despite the maximum temperature on the W canopy side, grapes grown on the two canopy sides of an N-S oriented row were rather homogenous compared to grapes from E-W oriented rows, in which the maximum possible difference of radiation occurred between the two canopy sides. In the E-W row orientation, as well as in NE-SW oriented rows, differences in grape phenolic, amino acid and malic acid concentration were measured between canopy sides.

The microclimate also influences the optical properties of a berry, mainly because of their effects on chlorophyll and carotenoid synthesis and degradation and phenol accumulation. This has been demonstrated using VIS-spectrophotometry as well as a UV/VIS fluorometer. Sorting berries by their VIS spectra produced subsamples of significantly different composition. The main compositional differences were the concentration of monoterpenes amino acids and malic acid. The former occurred in higher concentration in subsamples with less green and more yellow/red color, while the latter two occurred in higher concentration in juice and wine from greener berries. The common factor behind these compositional and optical differences seems to be the berry microclimate, which is in accordance with our other studies. These facts may be technically exploited in targeted berry sorting operations for ultra premium winemaking.

Kapitel 8

Zusammenfassung

Die Wirkung des Mikroklimas auf Traubengesundheit und Traubenqualität wird in der Weinwissenschaft bereits seit über 100 Jahren diskutiert, als erste, anekdotische Ergebnisse über den Effekt der Entblätterung auf die Traubenqualität veröffentlicht wurden. In den 1960er und 70er Jahren gewann die Manipulation des Mikroklimas der Laubwand zunehmend an Bedeutung, da die Züchtung wüchsiger Unterlagen und Edelreiser, die Ausbringung großer Mengen von Mineraldüngern und die Neupflanzung von Weinbergen auf fruchtbaren Böden zu einer übermäßigen Wüchsigkeit der Rebanlagen führten. Deren Konsequenz waren zunehmend verdichtete Laubwände, welche durch ein ungünstiges Mikroklima das Auftreten von Pathogenen begünstigten und letztlich auch eine verminderte Traubenqualität zur Folge hatten. Die Entwicklung von Gerätesystemen zur maschinellen Entblätterung Anfang der 90er Jahre, welche schnell eine große Verbreitung in der weinbaulichen Praxis fanden, führte zu einer erhöhten Forschungstätigkeit im Bereich des Mikroklimas. Die Möglichkeit der maschinellen Entblätterung entkoppelte zudem die Durchführung von Entblätterungsmaßnahmen von der wenig arbeitsintensiven Zeit nach Veraison. Auch wenn bereits seit den 1970er Jahren einige Studien zu den mikroklimatischen Effekten auf die Traubengesundheit und –qualität durchgeführt wurden, lag der Fokus der meisten dieser Studien auf der Anthocyansynthese roter Sorten bei einem Entblätterungszeitpunkt um Veraison. Hingegen wurden andere Entblätterungszeitpunkte und die mikroklimatischen Effekte auf die Qualität weißer Traubensorten selten beachtet.

Ziel dieser Thesis war es daher, ein eingehenderes Verständnis der mikroklimatisch bedingten Effekte auf die Traubenqualität von Riesling (*Vitis vinifera* L.) zu verschiedenen Zeitpunkten der Beerenentwicklung zu gewinnen. Hierbei sollte eine anwendungsoptimierte Hochdurchsatz-FTIR-Analytik sowie weitere Qualitätsanalytik durchgeführt werden. Um die mikroklimatisch bedingten Effekte auf die Traubenqualität zu untersuchen, wurden drei Methoden zur Manipulation des Mikroklimas einer Traube angewandt: Die komplette Freistellung aller Trauben in der Traubenzone; die komplette Beschattung von Trauben mittels lichtundurchlässiger Boxen und die Ausnutzung der natürlichen mikroklimatischen Variabilität auf den

beiden Seiten einer Laubwand bei den verschiedenen Reihenorientierungen Nord-Süd (N-S), Ost-West (E-W) und Nordost-Südwest (NE-SW). Die Reihenorientierung hat einen starken Einfluss auf den Temperaturverlauf sowie die Aufteilung der Strahlung auf die beiden Laubwandseiten. So ist in einer exakt N-S orientierten Zeile die Strahlung und Temperatursumme auf beiden Seiten der Laubwand relativ gleich verteilt, während in einer E-W orientierten Zeile fast die gesamte Strahlung auf die Südseite der Laubwand trifft, die folglich auch deutlich höheren Temperaturen ausgesetzt ist als die Nordseite derselben Laubwand. Thermographische Messungen zeigten jedoch, dass die maximalen Beerentemperaturen auf der Westseite der N-S orientierten Reihen auftraten. Die Temperaturerhöhung betrug hier 15 °C über der Lufttemperatur und ca. 10 °C im Vergleich zu einer von einer Blattschicht beschatteten Traube auf derselben Laubwandseite. In N-S orientierten Reihen wurde auch die höchste Temperatur in der Laubwand gemessen. Die Trauben aus N-S orientierten Reihen waren jedoch im Vergleich zu den Trauben aus NO-SW oder E-W orientierten Reihen in Bezug auf ihre Konzentration an Äpfelsäure, Aminosäuren und Phenolen sehr homogen.

Der Effekt der Mikroklima-Manipulation war – unabhängig vom Versuchsaufbau – im Fall der Flavonole groß und im Fall der Flavanole und Hydroxyzimtsäuren eher gering. Letzterer Effekt konnte nur durch Unterschiede im Mikroklima kurz nach der Blüte induziert werden, während Flavonole als Reaktion auf Belichtung nahezu proportional zur UV-Einstrahlung und ohne zeitliche Verzögerung neu synthetisiert wurden. Bei einer Abdunklung der Trauben wurde die Synthese von Flavonolen komplett eingestellt. Zu allen Entwicklungsstadien akkumulierte sich ein spezifisches Muster von Flavonol-glycosiden, abhängig von der Aktivität der jeweiligen Flavonol-Glycosyltransferasen während der Beerenentwicklung.

Im Vergleich zur Synthese der Flavonole scheint die Synthese der Monoterpene der Rebe einem komplexeren Modell zu folgen. Obwohl sich die Hauptphase ihrer Akkumulation im Stadium der Vollreife abspielt, scheint der Zeitpunkt um Veraison, wenn die Expression der Terpenoidsynthasen ihr Maximum erreicht, für ihre Synthese entscheidender zu sein. Während der gesamten Reifephase beschattete Rieslingtrauben hatten zur Lese einen kaum nachweisbaren Monoterpengehalt, doch auch ein Entfernen der Boxen vor der eigentlichen Hauptphase der Akkumulation führte lediglich zu einem geringen Anstieg der Monoterpenkonzentration. Letztlich wies auch diese Variante nur 20 % der Konzentration der in den Kontrolltrauben akkumulierten Monoterpene auf, während der

Flavonolgehalt auf das Niveau der Kontrollvariante anstieg. Eine sehr späte Entblätterung führt folglich lediglich zu einer starken Erhöhung des Phenolgehaltes der Trauben, während lediglich geringe Effekte auf das Traubenaroma zu erwarten sind.

Auch auf die Konzentration der Aminosäuren im Most konnte ein starker Einfluss des Mikroklimas festgestellt werden. Die Beschattung der Trauben führte dabei zu einer Erhöhung ihrer Konzentration, während der Sonne ausgesetzte Trauben eine niedrigere Konzentration als Trauben der nicht entblätterten Kontrolle aufwiesen. Effekte auf die Konzentration der Aminosäuren waren bereits vor Veraison nachweisbar, deutlich stärkere Effekte wurden jedoch bei einer Manipulation des Mikroklimas nach Veraison beobachtet, da in dieser Phase die Hauptakkumulation der Aminosäuren in den Beeren stattfindet.

Das Mikroklima beeinflusst zudem die Konzentration von Pigmenten wie Phenolen, Carotinoiden und Chlorophyll, indem es sowohl deren Synthese anregt wie auch deren Abbau beschleunigt. Damit hat das Mikroklima auch einen Einfluss auf die optischen Eigenschaften der Trauben. Messungen mit einem VIS-Spektrophotometer und einem UV/VIS Fluorometer, an den Proben verschiedener hier vorgestellter Studien, konnten in Kombination mit multivariater Datenanalyse Trauben valide zu unterschiedlichen Mikroklimaten zuordnen. Mit einer Sortierung im VIS-Spektralbereich konnten so Beeren in Klassen mit signifikant unterschiedlicher Ausprägung verschiedener Qualitätsmerkmale sortiert werden. Die so sortierten Untergruppen unterschieden sich in ihrem Gehalt an Monoterpenen, Aminosäuren im Most und Äpfelsäure in Most und Wein. Die Art und Ausprägung dieser Qualitätsunterschiede weist deutlich auf die mikroklimatischen Bedingungen als deren Ursache hin. Diese Tatsache kann in technischen, gezielten Sortierprozessen genutzt werden, um Beeren mit gewünschten Qualitätsmerkmalen für hochpreisige Weine zu selektieren oder unerwünschte Beeren aus großen Partien zu entfernen.

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Appendix

Overview of methods

The methods utilized for the generation of data introduced in this thesis will be shortly summarized in the following section.

Experimental vineyards

Field experiments for all trials except for the trial in Chapter 5 were conducted in the growing seasons 2010-2013 using Riesling (clone Gm 198-25; grafted to rootstock *Vitis berlandieri* Planch. x *Vitis riparia* Michx. cv. 'SO4', clone Gm47) in an established vineyard located close to Geisenheim, Germany (49° 59'20'' N; 7° 55'56''E). Vines were cane pruned and trained to a vertical shoot positioning (VSP)-type canopy system in three different row orientations: north-south (Row azimut 164°, N-S), east-west (Row azimut 254°, E-W) and southwest-northeast (Row azimut 209°, NE-SW). Row and vine spacing was 2.10 m and 1.05 m, respectively. In order to obtain a homogenous canopy, the shoot number was adjusted to ten - twelve shoots per vine. With the exception of the row orientation experiments, all trials have been conducted in the N-S orientation.

Grapes for the trial outlined in Chapter 5 were collected from an established vineyard of the Hochschule Geisenheim University, Germany (Geisenheimer Kläuserweg, approx. 49° 59'20'' N; 7° 55'56''E) planted with *V. vinifera* L. cv Riesling (clone Gm 198 grafted to rootstock *Vitis berlandieri* Planch. x *Vitis riparia* Michx. '5C'). The soil is deep, calcareous, with a predominance of clay and some marl. The vineyard has a steep slope and is south facing. Vines were cane pruned and trained to a vertical shoot positioning (VSP)-type canopy system in north-south (N-S) row orientation.

Sample preparation

Juice samples were mechanically pressed in a pressure controlled sample press at 1 bar (Longarone 85, QS System GmbH, Norderstedt, Germany) for 5 minutes. Pressing was stopped briefly after 2 minutes to allow stirring of the grapes. After pressing, the samples were filtered through a 16 µ Munktell 33/N folded filter (90 g m^{-2}; Ahlstrom, Helsinki, Finland) prior to analysis.

For berry skin analysis, the berries were cut off with their pedicel and stored immediately under CO_2 atmosphere and frozen at -20 °C. Berries were peeled under a CO_2 atmosphere whilst frozen. Skins were then freeze dried, ground with mortar and pestle and stored in an exsiccator until analysis.

Leaf samples consisted of leaf blades with the petiole removed. After sampling, leaf blades were immediately washed in distilled water, dried to constant weight at 60 °C and ground to a fine powder with a FOSS Cyclotec 1093 mill (FOSS, Hillerød, Denmark). Samples were stored in an exsiccator until analysis.

Berry skin analysis

For HPLC analysis of phenolics from berry skin, phenolic compounds were extracted from 0.1 g of the freeze dried grape skin powder in acidified acetonitrile under SO_2 protection followed by vacuum distillation of the extracts. The extracts were analyzed by an ACCELA HPLC/DAD system coupled to a LXQ mass spectrometer (ThermoFisher, Dreieich, Germany). Chromatographic separation was achieved on a 150x2 mm i.d., 3 µm Luna 3u C18 100A column (Phenomenex, Torrance, U.S.) protected with a guard column of the same material. Injection volume was 3 µL, at a flow rate of 250 µL/min. Elution conditions were: solvent A was 2 % acetic acid; solvent B was acetonitrile/water/acetic acid (50:50:0,5; v/v/v). Gradient elution was applied: 0-20 min from 96-50 % solvent A, 4-50 % solvent B, 20-23.1 min to 100 % B; washing with 100 % B for 2 min before re-equilibrating the column. Detection wavelengths were 280 nm for flavanols, 320 nm for phenolcarbonic acids and 360 nm for flavonols. The following mass spec conditions were used: ESI source voltage -3.00 kV during negative and +5.00 kV during positive ionization mode; capillary temperature 275 °C; collision energy for MS^n-experiments 35 % (arbitrary units). Peak identification was based on a combination of HPLC retention time and UV spectra as well as mass spectral data. Quantification was carried out using peak areas from external calibration curves. A table containing all standard sources is presented as supplemental table 1. Where no standards were available, substances were quantified using the calibration for the closest phenolic relatives (caftaric acid as caffeic acid; fertaric acid as ferulic acid, coutaric acid and p-CGT as coumaric acid).

Wine analysis

Total iron reactive phenolics in wine were measured according to the Harbertson-Adams assay (Harbertson and Spayd, 2006). In brief, wine samples were mixed with a TEA/SDS buffer (containing 5 % TEA (v/v) and 10 % SDS (w/v) adjusted to pH 7.9) in a microcuvette. Background absorbance of the solution was read at 510 nm after 10 min, and again after the addition of 125 µL Ferric chloride reagent (10 mM FeCl3 in 0.01 N HCl). All measurements were performed in duplicate and averaged. Total iron reactive phenolics concentration was calculated from a standard curve as catechin equivalents.

The aroma compounds extraction for the analysis of monoterpenols and norisoprenoids in chapter 5 was conducted using a protocol modified from Günata et al. (1985) and Kotseridis et al. (1998). 40 mL of wine were diluted with 40 mL of deionized water (50 %) and 8 µL of an internal standard (octan-3-ol 50 µg L^{-1}, DMH 25 µg L^{-1}) was added and passed through SPE-cartridge (Strata SDB-L, 500 mg Styrene-DivenylbenzenPolymer, Phenomenex, Torrance, USA) previously activated using 5 mL of Pentan/DCM (2:1, (v/v)), 10 mL of MeOH, 10 mL of MeOH / H_2O (1:1, (v/v)) and 10 mL of H_2O. The cartridges were flushed using 50 mL of water and dried for 30 minutes under nitrogen flow (100 mL min^{-1}). Then, the free aroma compounds were eluted by Pentan/DCM (2:1 (v/v); 5 mL, 3 mL). The extract was dried using anhydrous Na_2SO_4 and concentrated to 100 µL using a Vigreux column at 42 °C. Extracts were stored at -18 °C until analysis. The glycoside fraction was eluted using EtAc, solvent was removed using a rotation evaporator (40 °C; 138 kPa; 75 rpm) to dryness. The residue was dissolved in 7 mL of 0.2 M citric acid (pH: 2.5). Glycosides were hydrolyzed exactly for 1 hour at 100°C. Internal standard solution was added resulting in 50 µg L^{-1} DMH and 100 µg L^{-1} 3-Octanol related to initial sample volume and liquid-liquid extraction was conducted, using Pentan/DCM (2:1, (v/v); 2 mL, 1 mL, 1 mL). After drying with anhydrous Na_2SO_4, extracts were concentrated to 100 µL under gentle nitrogen flow (50 mL min^{-1}) and stored at -18 °C until analysis.

Extracts were analyzed using a Thermo Fisher Trace GC Ultra gas chromatograph equipped with a PTV Injector and coupled to a Thermo Fisher ITQ 900 Ion Trap MS mass spectrometric detector (all Thermo Fisher, Darmstadt, Germany). Gas chromatographic separation was carried out using a 30 m x 0.25 mm ID x 0.5 µm film thickness Agilent DB-Wax capillary column (Agilent Technologies, USA). 1 µL of the sample extract was injected in splitless mode (splitless time 90 s) at a temperature of 220 °C. The column

oven was held at 40 °C for 1 min during injection and then ramped to 60°C at 10 °C min^{-1}, from 60 °C to 200 °C at 3 °C min^{-1} and from 200 to 230 °C at 10 °C min^{-1}, which then was held for 10 min. The carrier gas was helium with a constant flow of 1.4 mL min^{-1} and an average velocity of 27 cm s^{-1}. The interface and MS source temperature were set to 240 °C and 200 °C respectively and mass spectrometric data were acquired in electron impact mode (EI) with ionisation energy of 70 eV. Selected ion monitoring mode (SIM) was used throughout each sample run with selected ions being used for the quantification of each aroma compound during post-run data analysis.

Juice analysis

Juice mineral nutrient content was analyzed by inductively coupled plasma with optical emission spectroscopy (ICP-OES, Spectro Arcos, Spectro Analytical Instruments GmbH, Kleve, Germany). All samples were analyzed in duplicate and the results averaged.

Juice total nitrogen content were analyzed by a modified Kjeldahl-digestion with ammonia determination by flow injection analysis (FIAstar 5000, Foss, Denmark) using photometric detection at 720 nm (Persson et al., 2008).

The concentration of primary amino acids in juice was determined according to the N-OPA procedure of Dukes and Butzke (1998). In brief, the amino acid groups were derivatised with o-phthaldialdehyde/N- acetyl-L-cysteine (OPA/NAC) reagent and absorbance at 335 nm was measured with a UV/VIS spectrometer (SPECORD 500, Analytik Jena AG, Jena, Germany) against a juice blank. Results were calculated as mg isoleucine equivalents from a standard curve.

Berry amino acids were analyzed with an amino acid analyzer S433 (Sykam, Eresing, Germany). Chromatographic separation was achieved on a 4.6 x 150 mm LCA K 07/Li cation-exchange column (Sykam) with post-column ninhydrin derivatisation and photometric detection at 570 and 440 nm for primary and secondary amino acids.

For the analysis of monoterpenols by gas chromatography – mass spectrometry in chapter 4, 100 g of crushed berries were thawed over night at 4 °C, mixed with a household blender and centrifuged for 10 min at 10° C and 4,600 rcf. The supernatant was carefully decanted from the centrifugation vessel. 40 mL of the juice were clarified with 5 mL Carrez I (150 g Potassium hexacyanoferrate (II) trihydrate/L) and 5 mL Carrez II (300 g Zinc sulphate

heptahydrate/L) solution, filled up to 100 mL and filtered (0.45 µ). Monoterpene analysis was conducted by SPE-GC/MS, following a method similar to the one of Di Stefano (1991). 2-octanol was used as internal standard (IST: 30 mg 2-octanol/ 100 mL CH_2Cl_2). SPE-cartridges (STRATA-X, Phenomenex, Torrance, CA) were conditioned with 10 mL CH_2Cl_2, followed by 5 mL MeOH and equilibrated with 5 mL H_2O. 30 mL clarified juice were added on prepared cartridges, followed by 10 mL distilled H_2O. Cartridges were dried under air flow for 30 min. Free monoterpenes were eluted with 10 mLCH_2Cl_2. 25 µL of internal standard were added and residual water removed by adding anhydrous sodium sulphate. Extracts were concentrated in a vacuum evaporator (Büchi, Flawil, Switzerland) at 30° C and 520 mbar. The concentrate was transferred to a 200 µL GC vial inlet and used for analysis. Glycosylated monoterpenes were eluted with 5 mL MeOH and desiccated in a vacuum evaporator at 45° C and 100 mbar. The dried methanol eluent was resuspended with citrate buffer (5 ml, pH 4.0) and 25 mg β-glycosidase (VP 1050-1, Erbslöh AG, Geisenheim, Germany) were added. Reaction time was 12 h at room temperature in the dark. After glycosidase reaction, 2 mL MTBE were added and the mixture was vortexed for 30 s and subsequently centrifuged (room temperature, 1600 rcf, 10 min). The supernatant was transferred to a 200 µL GC vial inlet and used for analysis.

Gas chromatographic analysis was conducted with a 6890 N Network GC System coupled to a 5973 N mass spectrometer (Agilent technologies, Santa Clara, U.S.) with a 30.0 m x 250 µm ID x 0.25 µm ZB-WAX column (Phenomenex, Torrance, U.S.). Sample injection volume was 1 µl in split/splitless mode (split ratio 10:1) at a temperature of 230 °C. The temperature program was as follows: 40 °C, held for 2 min, followed by 4 °C min^{-1} to 220 °C, held for 15 min. Sample injection was conducted with a MPS 2 autosampler (Gerstel, Mühlheim, Germany). The carrier gas was helium with a constant flow of 1.3 mL min^{-1}. The interface and MS source temperature were 280 °C and 230 °C respectively. Data were acquired in full scan mode (m/z 30-300). A more detailed description of the method has been published by Nitsch (2013). Quantification was performed using peak area of TIC (total ion current) and a response factor calibration with the respective monoterpene standard solutions. Analytical standards of pyran linalool oxides and diendiol I were not available. A response factor (Rf) of 1 was used for these compounds (unit of measurement: "µg L^{-1} Rf^{-1}").

FTIR spectra aquisition

From the data collected in chapter 2 (Friedel et al., 2013), it was concluded to use the FT2 Winescan (FOSS, Hillerød, Denmark) instrument for larger sample volumes and the OenoFoss™ instrument from the same manufacturer when sample volumes were limited, e.g. when analyzing wine from microfermentations or samples comprising 1-20 berries, as sample volume for this instrument is only 400 µL compared to about 40 mL for a double measurement on the FT2 Winescan. The analysis of the spectra is described under 'mulitvariate data analysis'.

Leaf analysis

0.25 g of leaf sample were acid digested following the procedure described by Döring et al. (2015). Leaf mineral content in the extracts was analyzed by inductively coupled plasma with optical emission spectroscopy (ICP-OES, Spectro Arcos, Spectro Analytical Instruments GmbH, Kleve, Germany). Nitrogen in the leaf extracts was analyzed by a modified Kjeldahl procedure in the same way as juice nitrogen. All samples were analyzed in duplicate and the results averaged.

Berry optical traits

Reflectance in the visible (VIS) part of the spectrum of the berry surface was measured from two opposite sides of each berry with a spectrophotometer (Konica Minolta 3500d, Tokyo, Japan), using a 3 mm aperture. Data were either exported as reflectance spectra or as color data using the CIE L*a*b* color space (Lübbe, 2012). The parameters lightness (L*), red/green (a*), and yellow/blue (b*) were calculated using the D65 illuminant and a 10 degree standard observer.

Fluorescence measurements were conducted with a Multiplex® MX 3.6 (Force-A, Paris, France) portable 'polyphenolmeter'. Bunches were destemmed and berries of every bunch were measured on a tray supplied by the manufacturer (Multiplex® Lab set). The measurements were corrected with correction tables supplied by the manufacturer and checked for measurement outliers by the procedure specified by the manufacturer.

Microclimate measurements

Bunch surface temperatures were measured by infrared thermography (H2640, NEC Avio Infrared Technologies, Tokyo, Japan). RGB and Infrared images were downloaded and analyszed in the InfRec Software (NEC Avio). Emissivity was set to 0.98. Images were automatically overlaid, bunches were marked and min, max and average temperatures of the marked areas of an image were exported to Microsoft Excel for further analysis.

Temperature and humidity were monitored by placing temperature probes (EasyLog *EL*-USB-2, LASCAR, UK) inside the canopy at the height of the bunch zone. Readings were taken every five minutes.

Incident radiation was measured by inserting LI-190 SA50 Quantum Sensors (Li-Cor, Lincoln, USA) connected to a LI-1400 data logger inside the canopy.

Solar radiation absorbed in the bunch zone was assessed using a radiation distribution model based on tracing of a random sample of photons in conjunction with the Monte Carlo method. The model was based on the grapevine radiation model published by Hofmann et al. (2014) and adapted to calculate only the radiant energy density absorbed by the area of the bunch zone in which sampling was conducted (80-110 cm above ground, western side of the canopy). For the calculations, diffuse and global radiation data supplied by the german weather service (DWD) station at Geisenheim, located approximately 2 km from the experimental vineyard, were used.

Univariate data analysis

The univariate statistical analysis of the results of chapter 5 was conducted using the open source R 3.0.1 statistical computing environment (R Development Core Team, 2006). Data analysis computed on R comprised one-way-analysis of variance (ANOVA) and two-way-ANOVA with a post-hoc Tukey HSD test.

All other univariate statistical calculations were conducted using SPSS 15.0 software (IBM, Armonk, U.S.). Apart from ANOVA, ANCOVA was used in cases in which an effect on the analyte of co-varying factors like sugar concentration was expected (e.g. chapter 4).

In chapter 3, experimental results were evaluated using a generalized linear model (GLM) for normally distributed data with treatment, year and sampling

date as factors. Post-hoc pairwise comparisons were performed by a Fisher's LSD test

Multivariate data analysis

Principal component analysis (PCA) was conducted using the MatLab PLS Toolbox 'svd' algorithm (Eigenvector Inc., Eagle Rock, U.S.). Auto scaling was applied before calculating the model.

Partial least squares discriminant analysis preceded by variable selection (iPLSDA) was conducted on fluorescence and phenolic composition data of the single bunches at veraison and harvest. To validate the discrimination model, the data set was split in half for calculation of the model and model validation respectively. Autoscaling of the data was applied before calculating the iPLSDA. The Software used for the calculations of the iPLSDA was Matlab® (The Mathworks, Natick, U.S.A.) employing the PLS toolbox (Eigenvector Inc., Eaglerock, U.S.A.) SIMPLS algorithm.

PLS regression for analysis of FTIR spectra

For the analysis of FTIR spectra, spectra from OenoFoss™ an FT2 WineScan were converted to be used in the OPUS software (Bruker, Ettlingen, Germany) with a Matlab (The Mathworks, Natick, U.S.A.) conversion routine. Performing calibrations in OPUS was preferred to the use of MatLab due to the easiness of data handling and calibration, while offering a number of good data processing tools for calibration. Data were analyzed using a "guided" iPLS algorithm, in which the respective intervals to be used in the calibration could be defined by the user. For skilled users, the utilization of this algorithm allows to discard noisy variables *a priori* and select narrow intervals in spectral regions in which information is dense, and wide intervals in spectral regions with less information, thus minimizing calculation time for calibration. The software further allows for the simultaneous testing of several data preprocessing algorithms such as 1st and 2nd derivatives or offset corrections in the calibration process.

The database for juice and wine calibrations on the FT2 comprises samples starting from 2008. Juice samples were sourced from all departments of Hochschule Geisenheim University and comprised different cultivars, degrees of bunch rot and ripeness levels. Reference data were obtained from the laboratory of the department of wine chemistry and beverage technology.

The calibration was updated yearly when the reference data were available (typically December/January) by including half of the novel reference data in the calibration and using the other half for a slope/intercept adjustment of the new calibration. The spectra of the latest harvest were then analyzed *a posteriori* with the new calibration. The calibration procedure for the OenoFoss™ was identical to the one of the FT2 Winescan, with the exception that it started only in 2009.

Calibrations for juice analysis on both instruments comprised TSS, glucose, fructose, TA, pH, malic acid and glycerol. Form 2013 onwards, enough reference data were available to include tartaric acid concentration in the calibration. Wine calibrations comprised the parameters Glucose, Fructose, TA, malic acid, lactic acid, pH, glycerol, ethanol and tartaric acid from 2013 onwards.

Personal contributions to the research papers in Chapters 2-5

Chapter 2: Matthias Friedel, Claus-Dieter Patz, Helumt Dietrich (2013). Comparison of different measurement techniques and variable selection methods for FT-MIR in wine analysis. Food Chemistry **141**, 4200-4207.

Status of the manuscript: published

Own contribution: All FT-MIR measurements, all calibrations except GA-PLS, validation, statistics, writing

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Chapter 3: Matthias Friedel, Manfred Stoll, Claus-Dieter Patz, Frank Will, Helmut Dietrich (2015). Impact of light exposure on fruit composition of white 'Riesling' grape berries (*Vitis vinifera* L.). *Vitis* **54**, 107-116

Status of the manuscript: published

Own contribution: Design of the field experiment, measurements, sampling, laboratory analysis (Phenolics by HPLC: Frank Will), statistics, writing

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Chapter 4: Matthias Friedel*, Johanna Frotscher*, Matthias Nitsch, Marco Hofmann, Jochen Bogs, Manfred Stoll, Helmut Dietrich (2016). Light promotes expression of monoterpene and flavonol metabolic genes and enhances flavour of winegrape berries (*Vitis vinifera* L. cv. Riesling). Australian Journal of Grape and Wine Research **22**, 409-422

Status of the manuscript: published

Own contribution: Conception and design of the field experiment (with Jochen Bogs), implementation of the field trial, sampling, field measurements, qPCR (with Johanna Frotscher), sample preparation and laboratory analysis (GCMS: Matthias Nitsch), statistics, writing

*) shared authorship

Chapter 5: Matthias Friedel*, Vincenzo Sorrentino*, Magali Blank, Armin Schüttler (2016). Influence of berry diameter and colour on some determinants of wine composition of *Vitis vinifera* L. cv. Riesling. Australian Journal of Grape and Wine Research **22**, 215-225

Status of the manuscript: published

Own contribution: Design of the experiment (with Magali Blank), field sampling, implementation of the trial (with Vincenzo Sorrentino and Magali Blank), laboratory analysis (with all co-authors), statistical analysis, writing

*) shared authorship