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Vitis **54** (Special Issue), 173–174 (2015)

Research Note

Advances in grape phenotyping: pigment characterization by reflectance and theoretical chemistry

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Key words: grape color; optical properties; anthocyanins; chlorophylls; melanin-like pigments.

Introduction: Despite the significant developments over the last decades in molecular biology through the huge production of genetic data, phenotypic records are missing. To complete the biological understanding of the *Vitis vinifera* L. genetic code expression, a thorough phenotypic description is mandatory. Due to the variability of the trait natures (e.g. phenology, chemical composition, plant morphology), phenotyping requires the development of specific methodologies. These methods also ideally need to be fast and high-throughput, namely data collection in a short time (following plant phenology) and possibility of replication (in relation to the natural variability particularly due to environmental effects and genetic *per* environmental (*GxE*) interactions).

Over the past years, our research group has decided to focus on non-invasive methods based on pigment optical properties. The color of individual molecules results from the absorption in a specific spectral range and related reflection, and transmission phenomena at these specific wavelengths. As an example, thanks to the π-delocalization over the entire flavylium cation of anthocyanins, green light is absorbed, resulting in red color (Allen 1998). From the optical property rationalization of pigments, it is possible to dissect the reflectance spectra obtained by non-invasive measurements, recognizing the absorption bands of the different molecules. In this way, it will be possible to develop algorithms able to describe the chemical composition of fruits.

In the framework of the COST Action FA1003 - Grapenet: East-West Collaboration for Grapevine Diversity Exploration and Mobilization of Adaptive Traits for Breeding - our group has been particularly involved in the phenotyping research area. This note is a synthetic review of the optical studies of pigment characterization developed by our research team over the past years.

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Material and Methods: Pigment optical properties have been studied by theoretical chemistry and reflectance spectroscopy. Anthocyanin absorption was described by quantum chemistry calculations based on time-dependent density functional theory (TD-DFT). Further methodological details are reported in Rustioni *et al.* 2013a. Aiming at a grape color classification, berries were studied by a Lambda 35 UV-vis spectrophotometer (Perkin Elmer, Waltham, Massachusetts), completed by a Spectralon integrating sphere (as described in Rustioni *et al.* 2013b). To elucidate the sunburn physiology with the purpose of resistant cultivar selection, chlorophylls and melanin-like pigments were studied by a Jaz System spectrometer (Ocean Optics) (Rustioni *et al.* 2014).

Results and Discussion: The Figure shows the optical properties of grape pigments (anthocyanins), as studied by different methods. These pigments exhibit specific absorption bands in the visible range. Quantum calculations enable in-depth understanding of the electronic origin of these bands, based on the description of excited states from electronic transitions involving π-type molecular orbitals (Figure a). The choice of theoretical methods is critical and requires benchmarking on small and well-documented prototypes. An example of grape anthocyanins quantum study is reported in Rustioni *et al.* 2013a. From an experimental view, the absorbance spectra of malvidin-3-O-glucoside can be obtained after separation by HPLC (Figure b). Nevertheless, to be more consistent with grape molecular composition, we should consider that the berry skin is constituted by a mixture of anthocyanins (Figure c), in which malvidin-3-O-glucoside is the major constituent. Comparing Figure a, b and c, it is possible to observe slight differences of the maximum absorption wavelengths and of the shapes of the spectra, as evaluated by the different methods. These methods are relatively system- and environment- dependent e.g., the optical properties of the extracted anthocyanins are measured in extremely acid solvents able to stabilize the red flavylium cations. The reflectance allows a more adapted description of the actual fruit color, but the obtained spectra (Figure d) should be clean and accurate enough. Despite the complexity of grape pigment distribution (high anthocyanin concentration as observed by absorption saturation and presence of other pigmented molecules), a reflectance index of pigment concentration has been proposed by our group. Moreover, the berry colors were gathered based on reflectance spectra, producing an objective method in cultivar classification (Rustioni *et al.* 2013b).

From the reflectance evaluation, other pigments were also studied, aiming at a cultivar sunburn susceptibility evaluation. In this case, our attention was focused on the photo-oxidative role of chlorophylls and carotenoids. Some reflectance indexes already published were tested and others were developed to quantify these molecules (Rocchi *et al.* unpubl.). Melanin-like pigments, the brown phenolic oxidation products yielded under radiative stress,

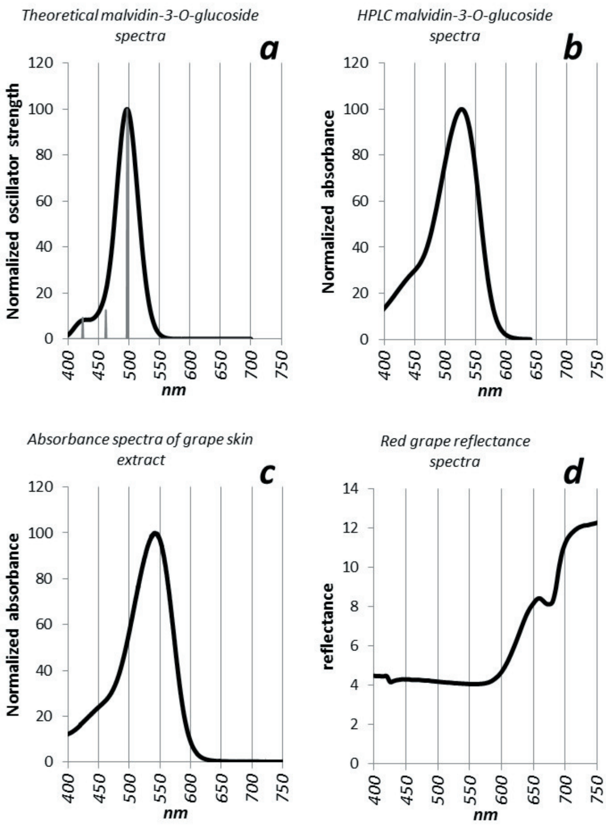

Figure: Optical properties of grape anthocyanins studied by different methods. **a**) Theoretical malvidin-3-O-glucoside spectra (the oscillator strength is directly correlated to the absorbance): the excited states are reported in gray and the black line indicates the obtained Gaussian function. **b**) Absorption spectra of malvidin-3-O-glucoside recorded after HPLC separation. **c**) Grape extract absorbance spectra measured by spectrophotometer. **d**) Reflectance spectra of red grape skin.

are particularly difficult to be measured due to their structural heterogeneity as well as difficulties in their extraction. The reflectance approach has allowed us the study of these polymers, developing a specific index. In this way, the sunburn symptoms can be objectively recognized and quantified.

Conclusions: The understanding of optical properties of grape pigments has allowed paving the way for the development of high-throughput non-invasive phenotyping methods. The main limitation of this approach is related to the difficult data elaboration, however the speed of data recording (typical of reflectance approach) is particularly adapted to analyze high number of cultivars during the short phenological phase of interest.

Joint publication of the COST Action FA1003 "East-West Collaboration for Grapevine Diversity Exploration and Mobilization of Adaptive Traits for Breeding".

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