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# Study of Sangiovese Wines Pigment Profile by UHPLC-MS/MS

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Supporting Information

**ABSTRACT:** The metabolic pigment composition of Sangiovese wines produced from grapes harvested at 20 different vineyards in Montalcino over three consecutive years (2008−2010) on a semi-industrial scale and of 55 commercial Brunello di Montalcino wines (2004−2007) was studied, using a targeted method capable of analyzing 90 pigments in an 11 min UHPLC-MS/MS chromatographic run. Interesting correlations were shown between various pigments formed during wine aging and those present in Sangiovese grapes. Vitisin B-like pigment and vitisin A-like pigment concentrations would seem to have a good correlation with ethyl-linked and direct-linked flavanol−anthocyanin concentrations, respectively. Moreover, the anthocyanic pattern recognition, genetically controlled by the plant variety, was shown to be inherited by the pigments formed during wine aging.

**KEYWORDS:** *UHPLC, tandem mass spectrometry, anthocyanins, traceability, wine aging, Sangiovese*

## INTRODUCTION

Sangiovese is a grape variety of considerable economic importance, used to produce some iconic wines, the most important being Brunello di Montalcino, produced in Montalcino (Siena, Tuscany), which can be considered the flagship of Italian enology. Today, 250 wineries produce almost 7 million bottles a year from 2100 ha, with a turnover of around 140 million euro. According to current regulations, DOCG (Denomination of Controlled and Guaranteed Origin) Brunello di Montalcino wines must be produced exclusively using Sangiovese grapes from the Montalcino production area and can be put on the market only in the fifth year after harvest, following a minimum of two years of aging in oak barrels (http://www.consorziobrunellodimontalcino.it).

One of the main characteristics distinguishing Sangiovese from other red wines is its delicate pigment profile.[1,2] Because color is one of the aspects determining quality in red wines, numerous scientific works have focused on trying to protect and enhance the color of Sangiovese.[1−5] The Sangiovese grape is not particularly rich in anthocyanins with respect to other cultivars[2,5] and contains rather high percentages (ca. 45% of the total) of unstable dihydroxy pigments, that is, cyanidin 3-glucoside, delphinidin 3-glucoside, and petunidin 3-glucoside, the concentration of which drops during winemaking with respect to that of methoxylated anthocyanins (peonidin 3-glucoside and malvidin 3-glucoside). The Sangiovese grape is also poor in acylated pigments. It should also be borne in mind that Brunello di Montalcino wines can be aged for a long time, improving as the years go by, from a minimum of 10 years to around 30 years, and excellent vintages can be kept for even longer. Superior color stability is therefore an essential requisite for this wine.

In addition to anthocyanins originally from the grapes, numerous pigments are formed during winemaking and the aging of wine. These include vitisin A-like pigments (e.g., vitisin A, **1**, in Figure 1), vitisin B-like pigments (e.g., vitisin B, **2**, in Figure 1), pinotin A-like pigments (e.g., pinotin A, **3**, in Figure 1), ethyl-linked flavanol−anthocyanins and direct-linked flavanol-anthocyanins, etc. (e.g., **4** and **5** in Figure 1).[6−12] Most theories on the formation of these pigments are based on model wine solution experiments.[8,13−18] Although this type of strategy has major limitations and drawbacks, it has delivered critical information about wine color stability and evolution over time. However, the full picture regarding the physicochemical and biological transformation occurring in the wine pigment profile is not clear, and there is little knowledge regarding any correlation between the various pigments.

Moreover, the anthocyanic profile is of great importance from a taxonomical point of view, because the pattern in grape is under strict genetic control, and although absolute concentrations can vary due to environmental and agronomical factors, the anthocyanic profile for each variety is relatively stable.[2,5,7]

For many years, HPLC-DAD instruments were the first choice for the analysis of anthocyanins because of their structural characteristics. Although the UV−vis spectra provide very useful information for quantitative and qualitative analysis, with high selectivity, repeatability, and efficacy in the measurement of anthocyanins, they also have a few limitations (sensitivity, time-consuming nature, etc). Therefore, in the past decade, and with the development of UHPLC systems, tandem mass spectrometry detectors have increasingly been used for pigment analysis because of their sensitivity and the possibility of analyzing a large number of analytes over a wide range of concentrations in a short period of time.[6,7,13,19−23] These characteristics of MS detectors give good promise for the future use of UHPLC-MS/MS systems in wine analysis, especially in the field of wines with long aging periods, which require one, two, or three decades to reach the market, in which these

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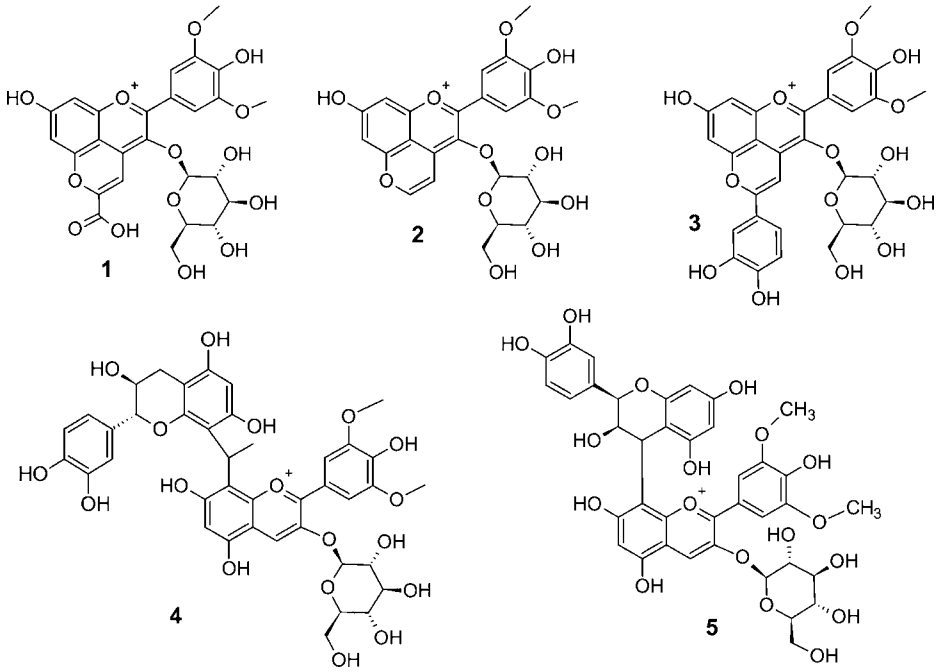


**Figure 1.** Chemical structures of typical pigments formed during wine aging: vitisin A (**1**); vitisin B (**2**); pinotin A (**3**); malvidin-3-glucoside-(8,8′)-ethyl-epicatechin (**4**); malvidin-3-glucoside-(4,8′)-catechin (**5**).

pigments are present in low amounts, often not detectable by conventional methods.

The objectives of this work were to develop a UHPLC-MS/MS method to study the composition of Sangiovese wines made from Montalcino zone grapes through detailed fingerprinting of the pigments and to follow the evolution and changes in these pigments taking place over time.

## MATERIALS AND METHODS

**Sampling and Winemaking.** Twenty *Vitis vinifera* L. cv. Sangiovese grape samples from different areas of Montalcino (Tuscany, Italy) were officially collected by the Italian Ministry of Agricultural, Food and Forestry Policy with the collaboration of inspectors from the Montalcino Consortium. A detailed sampling plan was prepared to trace every sample to the vineyard and to the plants sampled. The vineyards were chosen from the official geographic system ARTEA to cover the whole area of production of Brunello di Montalcino. From each selected vineyard, a total of ca. 80 kg of grapes was sampled in 20 kg boxes. Clusters (one per vine) were randomly picked from >150 vines, located in at least four noncontiguous rows exactly traced on the map of the vineyard, and sent in refrigerated transport (4 °C) to the experimental winery at the Edmund Mach Foundation (Trentino, Italy) on the same day.

The grapes were harvested on two separate occasions a week apart at the end of September in order to have samples at technological–enological maturity and being representative of 10 early- and 10 late-ripening vineyards in Montalcino. The grapes from each zone were vinified separately. For detailed vinification data see Table S1 of the Supporting Information. After malolactic fermentation, in March–April of each year, the young wine samples were filtered and bottled in 375 mL dark glass bottles and kept at controlled temperature and humidity levels until analysis. For detailed enological values of the bottled wine see Table S2 of the Supporting Information. The same procedure was repeated for three years (2008, 2009, and 2010), and all of the wines were analyzed together in February 2011. This allowed us to obtain a measure of the concentration of the pigments in 5-, 17-, or 41-month-old Sangiovese wines.

**Commercial Wines.** A sample set of 55 commercial Brunello di Montalcino wines from 19 different wineries and 4 different vintages (2004–2007) was also analyzed. Most of these were provided by the Consortium Brunello di Montalcino to cover the maximum possible variability of the Montalcino production area, whereas a few others came directly from wineries in the same area. These wines were analyzed in September 2011, thus allowing us to measure the concentration of the pigments in 4-, 5-, 6-, or 7-year-old Sangiovese wines.

**Chemicals.** All chemicals used in this study were of the highest purity grade available and purchased from Polyphenols Laboratories AS (Sandnes, Norway), unless otherwise stated. Methanol of LC-MS Chromasolv grade used for the preparation of mobile phase was purchased from Fluka Sigma-Aldrich. Methanol of Chromasolv HPLC grade used for the extraction and isolation was from Sigma-Aldrich. Ethyl acetate, diethyl ether, and ethanol were from Carlo Erba. Formic acid used as the mobile phase additive was of LC-MS grade from Sigma-Aldrich. Water purified by a Milli-Q water purification system was used for chromatography and preparation of standard solutions.

**Isolation.** For this study we selected the Serbina *V. vinifera* cultivar, considered to be autochthonous to Brescia (Italy). This is a rare variety, present in the Italian register of the Italian cultivars of grape for wine production with the Code 433; its cultivation is restricted to a small area in the region of Lombardia (Italy). Its anthocyanin pattern is unique as it consists mainly of *p*-coumaric esters.[24] The skins of 6 kg of ripe cv. Serbina grapes were extracted with 8 L of MeOH overnight, and then the extract was filtered to remove solid parts; its volume was decreased about 10 times by rotary evaporation under pressure at 30 °C and then diluted with $H_2O$ to a final volume of 8 L. The aqueous/methanolic solution was extracted (liquid–liquid extraction) twice with ethyl acetate, and the combination of the ethyl acetate extracts was evaporated to dryness using rotary evaporation under pressure at 30 °C and dissolved with 50 mL of MeOH.

Isolation of the anthocyanins was carried out in two steps. The instrument used was a Waters 2695 HPLC equipped with Waters 996 DAD detector (Waters Corp., Milford, MA, USA) and Empower Software (Waters). The eluents used were 0.3% $HClO_4$ in water (A) and MeOH (B), the injection volume was 50 $\mu$L, and the column oven temperature was 40 °C. For the first step a Supelco Discovery HS C18, 10 $\mu$m, 10 × 250 mm semipreparative column was used. The flow rate was 6 mL/min, and the gradient was as follows: from 72.5 to 64.5% of A for the first 4 min, from 64.5 to 55.5% A from 4 to 21 min, from 55.5 to 39.5% A from 21 to 30 min, and from 39.5 to 0% A from 30 to 30.1

**Table 1. UHPLC-MS/MS Identification Data**

| peak | $t_R$ (min) | MRM transitions[a] | cone voltage (V) | collision energy | identification[b] |
|---|---|---|---|---|---|
| 1 | 1.79 | 797→635 | 28 | 30 | mv glu GC |
| 2 | 1.90 | 737→575 | 28 | 30 | cy glu C |
| 3 | 2.06 | 767→605 | 28 | 30 | pt glu C |
| 4 | 2.20 | 751→589 | 28 | 30 | pn glu C |
| 5 | 2.37 | 611→287 | 38 | 52 | cy diglu[c] |
| 6 | 2.37 | 781→619 | 28 | 30 | mv glu C |
| 7 | 2.50 | 641→317; 641→302 | 28 | 30; 44 | pt diglu[c] |
| 8 | 2.56 | 595→271; 595→121 | 36 | 46; 60 | pl diglu[c] |
| 9 | 2.59 | 465→303; 465→229 | 20 | 22; 58 | dp glu[c] |
| 10 | 2.72 | 625→286; 625→301 | 34 | 60 | pn diglu[c] |
| 11 | 2.72 | 627→303 | 28 | 30 | dp caf glu |
| 12 | 2.81 | 781→619 | 28 | 30 | mv glu C |
| 13 | 2.81 | 533→371 | 28 | 30 | cpyr dp glu |
| 14 | 2.83 | 655→331; 655→315 | 40 | 42 | mv diglu[c] |
| 15 | 2.92 | 489→327 | 28 | 30 | pyr dp glu |
| 16 | 2.94 | 449→287; 449→137 | 28 | 28; 48 | cy glu[c] |
| 17 | 3.05 | 611→287 | 28 | 30 | cy caf glu |
| 18 | 3.17 | 479→317; 479→302 | 28 | 30; 42 | pt glu[c] |
| 19 | 3.41 | 433→271; 433→121 | 26 | 58; 36 | pl glu[c] |
| 20 | 3.51 | 463→301; 433→286 | 28 | 28; 42 | pn glu[c] |
| 21 | 3.51 | 547→385 | 28 | 30 | cpyr pt glu |
| 22 | 3.55 | 503→341 | 28 | 30 | pyr pt glu |
| 23 | 3.57 | 943→619 | 28 | 30 | mv pc glu GC |
| 24 | 3.62 | 493→331; 493→315 | 28 | 30; 50 | mv glu[c] |
| 25 | 3.74 | 589→385 | 28 | 30 | cpyr pt ac glu |
| 26 | 3.77 | 419→287; 419→137 | 26 | 52; 24 | cy arab[c] |
| 27 | 3.88 | 447→325 | 28 | 30 | pyr pn glu |
| 28 | 3.89 | 507→303; 507→229 | 30 | 30; 50 | dp ac glu |
| 29 | 3.96 | 531→369 | 28 | 30 | cpyr pn glu |
| 30 | 4.02 | 795→343 | 28 | 50 | pt glu et-C |
| 31 | 4.02 | 545→341 | 28 | 30 | pyr pt ac glu |
| 32 | 4.06 | 517→355 | 28 | 30 | pyr mv glu |
| 33 | 4.07 | 795→343 | 28 | 50 | pt glu et-C |
| 34 | 4.09 | 561→399 | 28 | 30 | cpyr ml glu |
| 35 | 4.15 | 649→487 | 28 | 30 | pyr pt pc glu |
| 36 | 4.20 | 779→327 | 28 | 50 | pn glu et-C |
| 37 | 4.20 | 927→619 | 28 | 30 | mv pc glu C |
| 38 | 4.22 | 433→301; 433→286 | 26 | 40; 22 | pn arab* |
| 49 | 4.28 | 491→287; 491→213 | 28 | 28; 54 | cy ac glu |
| 40 | 4.29 | 559→355 | 28 | 30 | pyr mv ac glu |
| 41 | 4.30 | 641→317 | 28 | 30 | pt caf glu |
| 42 | 4.30 | 779→327 | 28 | 50 | pn glu et-C |
| 43 | 4.32 | 825→367 | 28 | 50 | mv glu et-GC |
| 44 | 4.35 | 603→355 | 28 | 30 | cpyr mv ac glu |
| 45 | 4.40 | 529→325 | 28 | 30 | pyr pn ac glu |
| 46 | 4.42 | 521→317; 521→302 | 28 | 24; 46 | pt ac glu |
| 47 | 4.44 | 625→301 | 28 | 30 | pn caf glu |
| 48 | 4.45 | 611→303; 611→229 | 34 | 28; 54 | dp cpc glu |
| 49 | 4.46 | 693→385 | 28 | 30 | cpyr pt pc glu |
| 50 | 4.47 | 809→357 | 28 | 50 | mv glu et-C |
| 51 | 4.63 | 595→287; 595→136 | 34 | 34; 72 | cy cpc glu |
| 52 | 4.65 | 655→331 | 28 | 30 | mv caf glu |
| 53 | 4.69 | 597→435 | 28 | 30 | dp glu vc |
| 54 | 4.73 | 611→303; 611→229 | 34 | 28; 70 | dp pc glu[b] |
| 55 | 4.76 | 550→301; 505→286 | 28 | 30; 50 | pn ac glu[c] |
| 56 | 4.80 | 625→317; 625→302 | 34 | 28; 54 | pt cpc glu |
| 57 | 4.83 | 535→331; 535→315 | 30 | 26; 50 | mv ac glu[c] |
| 58 | 4.94 | 473→311 | 28 | 30 | pyr cy glu |
| 59 | 4.94 | 677→369 | 28 | 30 | cpyr pn pc glu |
| 60 | 4.94 | 707→399 | 28 | 30 | cpyr mv pc glu |
| 61 | 4.97 | 633→325 | 28 | 30 | pyr pn pc glu |


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**Table 1. continued**

| peak | $t_R$ (min) | MRM transitions[a] | cone voltage (V) | collision energy | identification[b] |
|---|---|---|---|---|---|
| 62 | 4.99 | 581→419 | 28 | 30 | dp glu vp |
| 63 | 5.00 | 663→355 | 28 | 30 | pyr mv pc glu |
| 64 | 5.02 | 595→287; 595→137 | 34 | 34; 72 | cy pc glu[c] |
| 65 | 5.12 | 609→301; 609→286 | 38 | 32; 54 | pn cpc glu |
| 66 | 5.12 | 625→317; 625→302 | 34 | 28; 54 | pt pc glu[c] |
| 67 | 5.14 | 851→357 | 28 | 50 | mv ac glu et-C |
| 68 | 5.16 | 639→331; 639→315 | 38 | 30; 58 | mv cpc glu |
| 69 | 5.21 | 611→449 | 28 | 30 | pt glu vc |
| 70 | 5.25 | 825→367 | 28 | 50 | mv glu et-GC |
| 71 | 5.26 | 743→435 | 28 | 30 | dp pc glu vc |
| 72 | 5.43 | 955→357 | 28 | 50 | mv pc glu et-C |
| 73 | 5.44 | 609→301; 609→286 | 28 | 32; 54 | pn pc glu[c] |
| 74 | 5.49 | 639→331; 639→315 | 38 | 30; 58 | mv pc glu[c] |
| 75 | 5.55 | 595→433 | 28 | 30 | pn glu vc |
| 76 | 5.57 | 595→433 | 28 | 30 | pt glu vp |
| 77 | 5.61 | 851→357 | 28 | 50 | mv ac glu et-C |
| 78 | 5.65 | 727→419 | 28 | 30 | dp pc glu vp |
| 79 | 5.75 | 625→463 | 28 | 30 | mv glu vc |
| 80 | 5.84 | 757→449 | 28 | 30 | pt pc glu vc |
| 81 | 6.02 | 667→463 | 28 | 30 | mv ac glu vc |
| 82 | 6.04 | 579→417 | 28 | 30 | pn glu vp |
| 83 | 6.16 | 609→447 | 28 | 30 | mv glu vp |
| 84 | 6.33 | 741→433 | 28 | 30 | pt pc glu vp |
| 85 | 6.34 | 639→477 | 28 | 30 | mv glu vg |
| 86 | 6.38 | 955→357 | 28 | 50 | mv pc glu et-C |
| 87 | 6.49 | 771→463 | 28 | 30 | mv pc glu vc |
| 88 | 6.50 | 651→447 | 28 | 30 | mv ac glu vp |
| 89 | 6.65 | 681→477 | 28 | 30 | mv ac glu vg |
| 90 | 6.96 | 725→417 | 28 | 30 | pn pc glu vp |
| 91 | 7.03 | 755→447 | 28 | 30 | mv pc glu vp |
| 92 | 7.12 | 785→477 | 28 | 30 | ml pc glu vg |

[a]When two transitions are indicated for a pigment, the first was the quantifier and the second the qualifier. [b]dp, delphinidin; cy, cyanidin; pt, petunidin; pn, peonidin; mv, malvidin; pl, pelargonidin; glu, 3-glucoside; arab, 3-arabinoside; diglu, 3,5-diglucoside; ac glu, 3-(6″-acetyl)-glucoside; pc glu, 3-(6″-*p*-coumaroyl)-glucoside *trans* isomer; cpc glu, 3-(6″-p-coumaroyl)-glucoside *cis* isomer; caf glu, 3-(6″-caffeoyl)-glucoside; pyr-, pyrano-; cpyr, carboxypyrano-; et-C, ethyl catechin or ethyl epicatechin; et-GC: ethyl gallocatechin or ethyl epigallocatechin; C, catechin or epicatechin; vp, 4-vinylphenol; vc, 4-vinylcatechol; vg, 4-vinylguaiacol. [c]Pigments identifed and quantified by standard.

min, followed by an isocratic hold for 2 min to clean the column; column equilibration was 10 min. Using this step cyanidin 3-(6″-*p*-coumaroyl)-glucoside and petunidin 3-(6″-*p*-coumaroyl)-glucoside were isolated. For the separation of malvidin 3-(6″-acetyl)-glucoside from delphinidin 3-(6″-*p*-coumaroyl)-glucoside, one more step was required. The HPLC system was the same, but the column used was a Purospher RP C18, 5 μm, 3 × 250 mm column. The flow rate was 1.1 mL/min, and the gradient was as follows: from 72.5 to 62.0% of A for the first 20 min and from 62.0 to 0% A from 20 to 20.1 min, followed by an isocratic hold for 2 min to clean the column. The same procedure was also required for the separation of peonidin 3-(6″-*p*-coumaroyl)-glucoside from malvidin 3-(6″-*p*-coumaroyl)-glucoside, with a small modification to the flow rate (0.9 mL/min) and the gradient (from 72.5 to 58.0% during the first 2 min, followed by an isocratic hold for 10 min with A brought to 0% for a 3 min column cleanup). The column equilibration for both runs was 5 min.

In all cases, automatic fractionation for collecting the pure peaks was carried out using a Fraction Collector III (Waters). Each solution was concentrated to dryness by rotary evaporation under pressure at 30 °C, then dissolved with 2 mL of ethanol, and precipitated by the slow addition of ethyl ether (7−8 times the volume of ethanol was required) and 2 N HCl (~0.1 mL). The precipitate was filtered on a membrane (0.22 μm) and dried in a desiccator under vacuum.

Petunidin 3-(6″-acetyl)-glucoside was isolated as described at Passamonti et al.[25]

The purity (>98%) and the identity of all isolated compounds were controlled by HPLC-MS/MS and HPLC-DAD analysis registered at 520 nm. The molar absorptivity ($\varepsilon$) of the isolated compounds was measured according to the method described by Dell'Agli et al.[26] Each standard used for the study was dissolved in the solution ethanol/water/HCl (70:30:1), at a pH value of 1.26. Two solutions were prepared containing, respectively, 5 and 10 mg/L of each compound. The spectral characteristics of the known solutions were recorded on a Hitachi U-2000 UV−vis spectrophotometer using quartz cells with a 10 mm optical path. The molecular weight used for molar absorptivity calculations included the weight of a chloride counterion. The average experimental values of the molar absorptivities of the commercial standards, measured at 520 nm, were in agreement with tabulated data.[26] The experimental molar absorptivities of newly isolated standards used in our experiments were found to be the following: for malvidin 3-(6″-*p*-coumaroyl)-glucoside, 22436 (540 nm); cyanidin 3-(6″-*p*-coumaroyl)-glucoside, 23172 (540 nm); peonidin 3-(6″-*p*-coumaroyl)-glucoside, 29843 (540 nm); petunidin 3-(6″-*p*-coumaroyl)-glucoside, 27211 (540 nm); delphinidin 3-(6″-*p*-coumaroyl)-glucoside, 31752 (540 nm); and malvidin 3-(6″-acetyl)-glucoside, 14219 (540 nm).

**Sample Preparation.** Sample preparation for the measurement of wine pigments was performed according to the method of Rossetto et al.[27] Briefly, 25 mL of wine diluted 4 times with $H_2O$ was applied to a C18-SPE cartridge (1 g, Waters), previously activated with MeOH (5 mL) and $H_2O$ (10 mL). The cartridge was washed with 6 mL of 0.3%

**Table 2. Calibration Data**

| analyte | regression curve $r^2$ | LOD[a] (μg/L) | LOQ[b] (μg/L) | linearity range (μg/L) | linearity orders |
|---|---|---|---|---|---|
| delphinidin 3-glucoside | 0.993 | 430 | 883 | 1765−176500 | 4 |
| cyanidin 3-glucoside | 0.993 | 107 | 355 | 1065−266250 | 3 |
| petunidin 3-glucoside | 0.986 | 116 | 387 | 1163−581650 | 4 |
| peonidin 3-glucoside | 0.968 | 3 | 9 | 93−92800 | 4 |
| malvidin 3-glucoside | 0.985 | 8 | 79 | 793−79300 | 3 |
| pelargonidin 3-glucoside | 0.999 | 10 | 31 | 31−31300 | 4 |
| peonidin 3-(6″-acetyl)-glucoside | 0.996 | 4 | 10 | 77−7700 | 3 |
| malvidin 3-(6″-acetyl)-glucoside | 0.991 | 16 | 80 | 1600−400000 | 3 |
| delphinidin 3-(6″-*p*-coumaroyl)-glucoside | 0.993 | 25 | 500 | 500−50000 | 3 |
| cyanidin 3-(6″-*p*-coumaroyl)-glucoside | 0.993 | 12 | 37 | 377−18550 | 3 |
| petunidin 3-(6″-*p*-coumaroyl)-glucoside | 0.988 | 23 | 46 | 913−91300 | 3 |
| peonidin 3-(6″-*p*-coumaroyl)-glucoside | 0.984 | 1 | 3 | 3−6260 | 4 |
| malvidin 3-(6″-*p*-coumaroyl)-glucoside | 0.990 | 3 | 9 | 22−43350 | 4 |
| peonidin 3,5-diglucoside | 0.998 | 11 | 33 | 33−6600 | 3 |
| malvidin 3,5-diglucoside | 0.997 | 48 | 120 | 120−120000 | 4 |
| peonidin 3-arabinoside | 0.999 | 5 | 13 | 13−5370 | 4 |
| cyanidin 3-arabinoside | 0.997 | 6 | 18 | 18−6250 | 4 |

[a]LOD, limit of detection (s/n = 6). [b]LOQ, limit of quantification (s/n = 10).

aqueous $HClO_4$ and then eluted with 10 mL of MeOH into a 100 mL flask. The elute was evaporated under reduced pressure at 30 °C, reconstituted in 1 mL of MeOH/$H_2O$ (1:1), filtered through 0.22 μm PTFE filters into a 2 mL autosampler amber LCMS certified vial (Waters), and injected. To avoid problems with sample stability, 10−12 samples were prepared per day so that all analyses could be completed within few hours after the sample preparation.

**Instrumentation and Methods.** Analysis of the samples studied was performed with an Acquity ultraperformance liquid chromatographic system (Waters, MA, USA) coupled to a Xevo TQ MS System (Waters, UK) operating under MassLynx XS software. All samples were analyzed on a reverse phase (RP) Acquity UPLC BEH C18, 1.7 μm, 2.1 × 150 mm column (Waters), protected with an Acquity UPLC BEH C18, 1.7 μm, 2.1 × 5 mm precolumn (Waters), at 40 °C and under a mobile phase flow rate of 0.4 mL/min. Water was used as weak eluting solvent (A) and methanol as strong elution solvent (B); formic acid 5% v/v was used as additive in both eluents. The multistep linear gradient used was as follows: from 95 to 60% of A for the first 4 min, from 60 to 45% A from 4 to 9 min, from 45 to 5% A from 9 to 11 min, and an isocratic hold for 3 min to clean the column. The equilibration time was 4 min, and the injection volume was 2 μL. All of the samples were analyzed in triplicate.

The column eluent was directed to the mass spectrometer, and analyte detection was performed by multiple reaction monitoring (MRM) using the MS/MS transitions shown in Table 1.

Electrospray positive ionization mode (ESI) was applied for all compounds with the parameters in the source set as follows: capillary voltage at 0.5 kV and block and desolvation temperatures at 150 and 500 °C, respectively. Desolvation gas flow was 1000 L/h and cone gas flow 20 L/h. LM resolutions were 2.55 and 2.80 for analyzers 1 and 2, respectively, whereas HM resolutions were 14.90 and 15.00 LM for analyzers 1 and 2, respectively. Ion energy for analyzer 2 was 1.0. The cone voltage and collision energy were optimum for each analyte (Table 1). The MRM conditions were optimized for the standards by direct infusion into the ES ionization source. For compounds for which standards were not available, the MRM transitions found in the literature were used and optimized through multiple injections of the same sample under various ion source parameters. Pigments have very similar structural characteristics to one another and are already charged, so their detection using a mass spectrometer occurs under very similar conditions. The chromatographic data reported in the literature were also used for identification.

**Quantification.** Quantification of individual compounds was performed using UHPLC-MS/MS, and the calibration data are reported in Table 2. When the authentic standard was not available, the analytes were quantified relative to malvidin 3-glucoside, by the malvidin 3-glucoside calibration curve.

**Data Analysis.** Data processing was performed using the Mass Lynx Target Lynx Application Manager (Waters). The precision/injection repeatability test (expressed as relative standard deviation, RSD, in %) was performed using a standard mix, made up of the *p*-coumaroyl derivatives of delphinidin, cyanidin, petunidin, and malvidin, which are more stable than simple glucosides. The standard mix was injected during batch analysis at least three to five times a day. These tests showed good precision in the peak area (RSD < 10%) for both intraday and interday. When the whole sample preparation (including sampling, dilutions, SPE, concentrations, and LC-MS analysis) was repeated in triplicate for two different wines, the coefficient of variation (CV) for the retention times was in a range of 0.2−0.4% and that for the concentration in a range between 0.4 and 13.0% (average = 3.25%).

## RESULTS AND DISCUSSION

Use of a modern UHPLC chromatography technique coupled with triple-quadrupole mass spectrometers to measure the various anthocyanins and their relative pigments allowed us to deal with a large number of analytes in a short analysis time, with a very effective and sensitive method. This matter is of considerable economic and scientific importance because the conventional HPLC method used for legal quality control of wine (OIV method adopted with the Resolution ENO 22/2003) does not provide sound results with aged wines and from an analytical point is clearly obsolete. The use of a 1.8 μm particle size column can significantly increase the analysis resolution in less time, whereas the quantification by a triple-quadrupole MS decreases the noise, allowing a noteworthy decrease of the limits of detection and quantification, in a larger linearity range, when compared with a DAD. It should be noted that both identification and quantification of all analytes were achieved with the MS detector. Because of these purposes it was possible to quantify all of the major wine pigments with no difficulties to wines aged for 7 years, which would be impractical for a DAD. At the same time it was possible to monitor 92 different pigments in a single run of 11 min.

Because it was impossible to have all of the analytes as authentic standards, a few compromises were made. When the standard was not available and thus it was not possible to

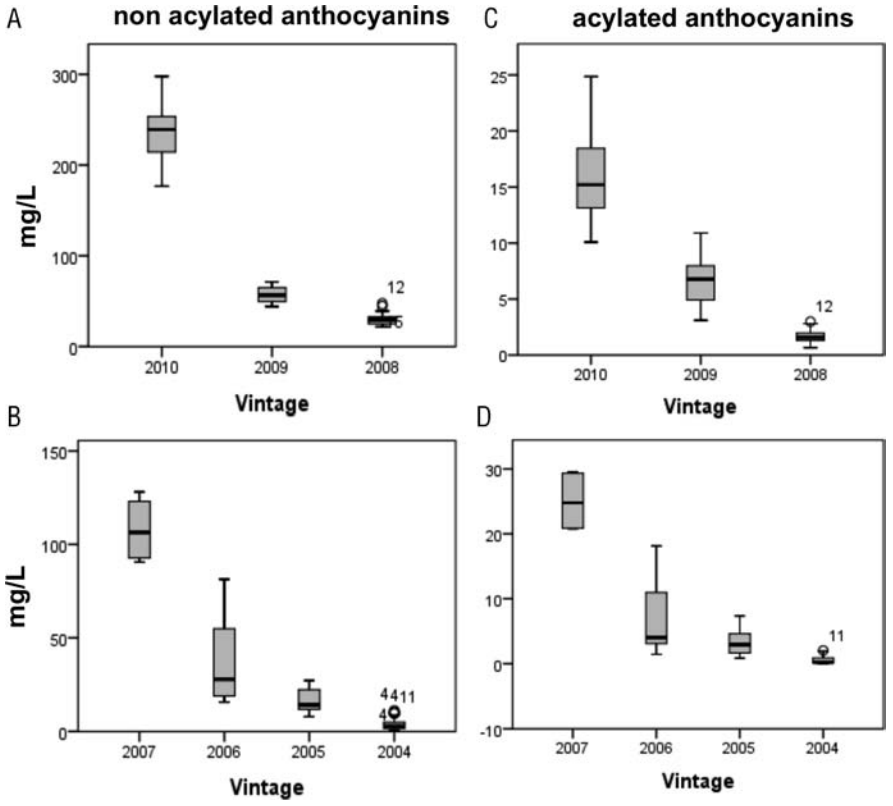


**Figure 2.** Plots A and B show the concentrations of the simple glucosides of malvidin, peonidin, petunidin, cyanidin, and delphinidin as sum (mg/L) with respect to the year of production (vintage); plots C and D show the concentration sum of their corresponding acetyl and *p*-coumaroyl esters.

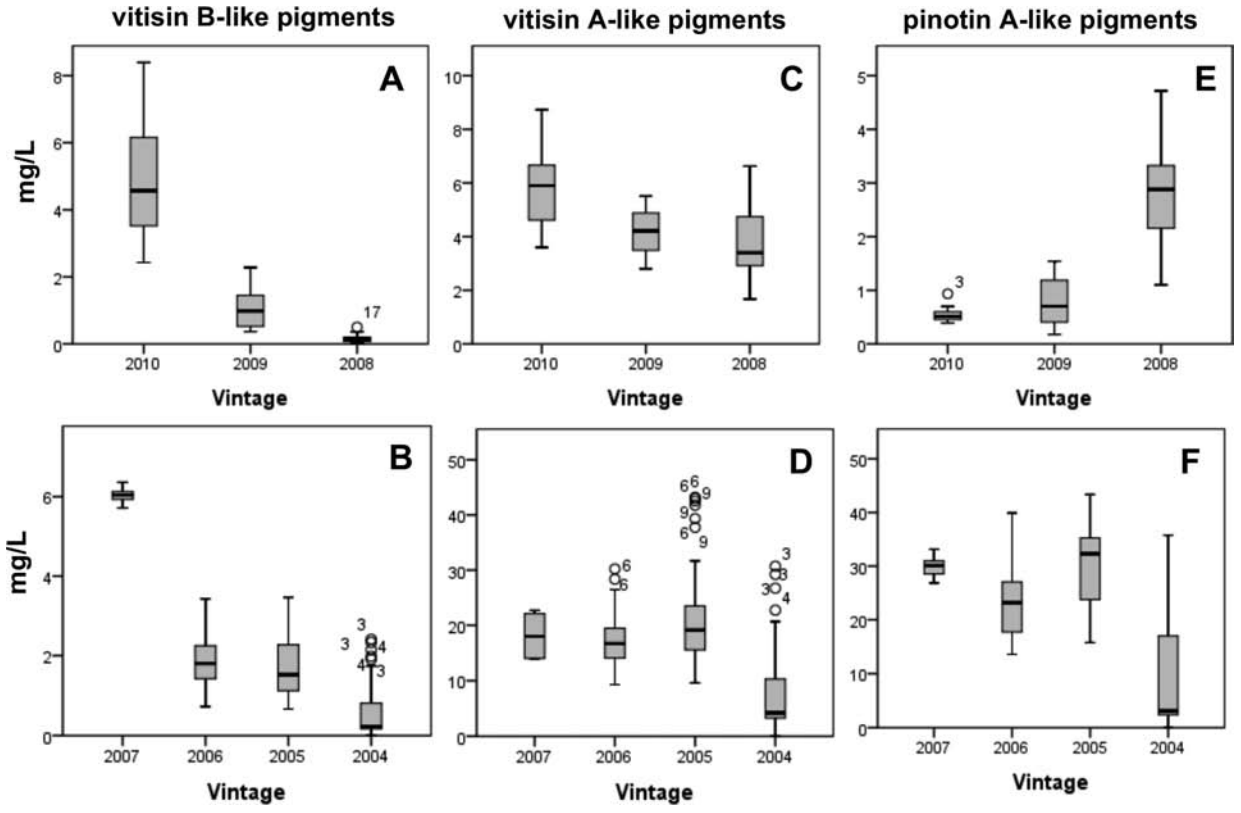


**Figure 3.** Plots A and B show the concentration sums of vitisin B-like pigments in the different vintages, plots C and D those of vitisin A-like pigments, and plots E and F those of pinotin A-like pigments. All concentrations are expressed as mg/L of malvidin 3-glucoside.


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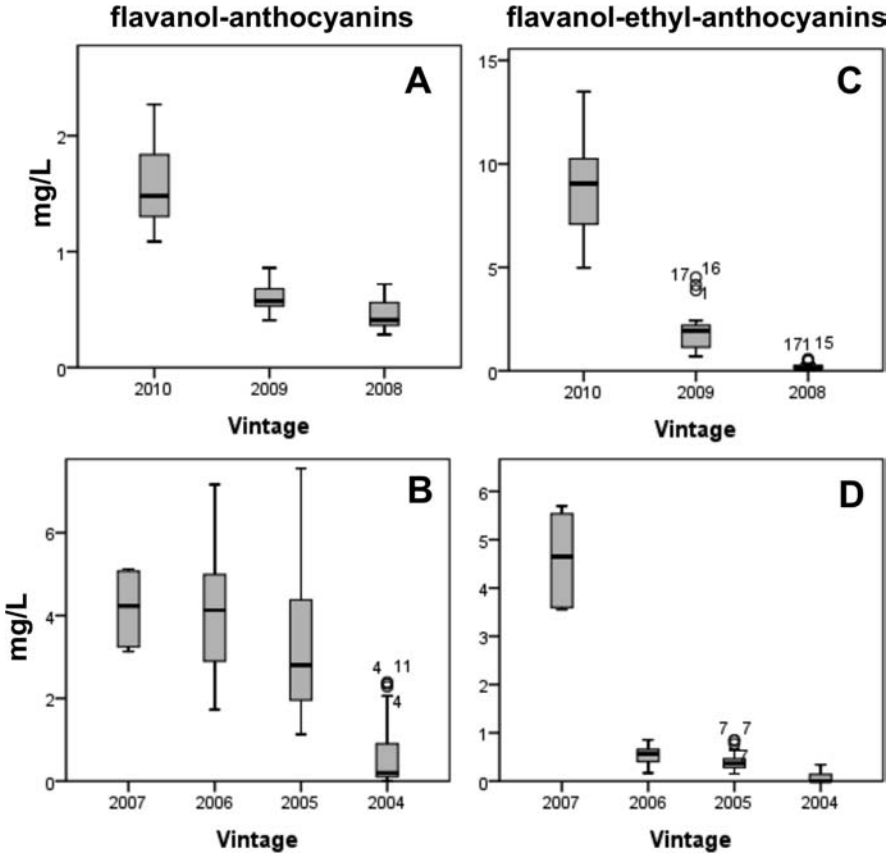


**Figure 4.** Plots A and B show the concentration sums of direct-linked flavanol−anthocyanins in the different vintages and plots C and D those of the ethyl-linked flavanol−anthocyanins. All concentrations are expressed as mg/L of malvidin 3-glucoside.

measure the absolute quantity, the amount of the analyte was expressed as a relative quantity by using the calibration curve of a similar compound or peak area. In the field of enology the first relative quantification method is more common, so in this work the compounds for which standards were not available were relatively quantified as equivalents of malvidin 3-glucoside, which is the main grape pigment. The main benefit of this technique is that it is easier to compare analyses carried out by different laboratories or methods or analyses carried out at different times.

A significant advantage of our experimental design was the opportunity to analyze the maximum biological variability of Sangiovese wines from grapes from the Montalcino area: 115 wines from 7 different vintages (2010−2004) and 20 different wineries were analyzed. The samples were divided in such a way that it was also possible to follow the variability of wines originating from the same vineyard and produced using the same technique from year to year. Although enological practices are restricted due to the rules regulating the production of Brunello di Montalcino DOCG, there are still many parameters that may change from one winery to another, which usually remain constant for each winery from one vintage to the next. In any case, our objectives did not include studying how enological methods influence wine pigments but rather focused on providing a broad overview and finding important pigment markers for Sangiovese wine color.

Because the winemaking techniques used in our experimental winery were different as compared to those used by the Brunello di Montalcino wineries (i.e., oak barrel aging), we decided to present the results in parallel graphs and not together.

The anthocyanins extracted from grape skin in the must during vinification, such as simple glucosides of malvidin, peonidin, petunidin, cyanidin, delphinidin, and their corresponding acylated derivatives, are found in relatively large quantities in young wines. However, the concentration of these compounds during wine aging gradually decreases due to their low stability in wine or due to their ability to participate in reactions leading to the formation of more stable pigments. For example, according to the literature vitisin A-like and vitisin B-like pigments are considered to be more stable than the corresponding grape anthocyanins.[28,29] In our experiment the absolute concentration of simple glucosides of anthocyanins was found to be much lower in the older wines, as expected (76% different between 2010 and 2009 and 87% between 2010 and 2008) (Figure 2); vitisin B-like pigments also showed a similar tendency, but with a slight faster speed (80% different between 2010 and 2009 and 97% between 2010 and 2008) (Figure 3A), whereas vitisin A-like pigments were more or less stable, not only in the three vintages of the experimental wines (26% different between 2010 and 2009 and 36% between 2010 and 2008) but also in the four vintages of commercial wines (Figure 3C,D). As expected for pinotin A-like pigments, their concentration was found to increase as the age of the wines increased (Figure 3E). The concentrations of these pigments were relatively similar in the various commercial wines (Figure 3F). This finding is in agreement with the paper of Rentzsch et al.,[30] in which pinotin A-like pigments showed a strong increase

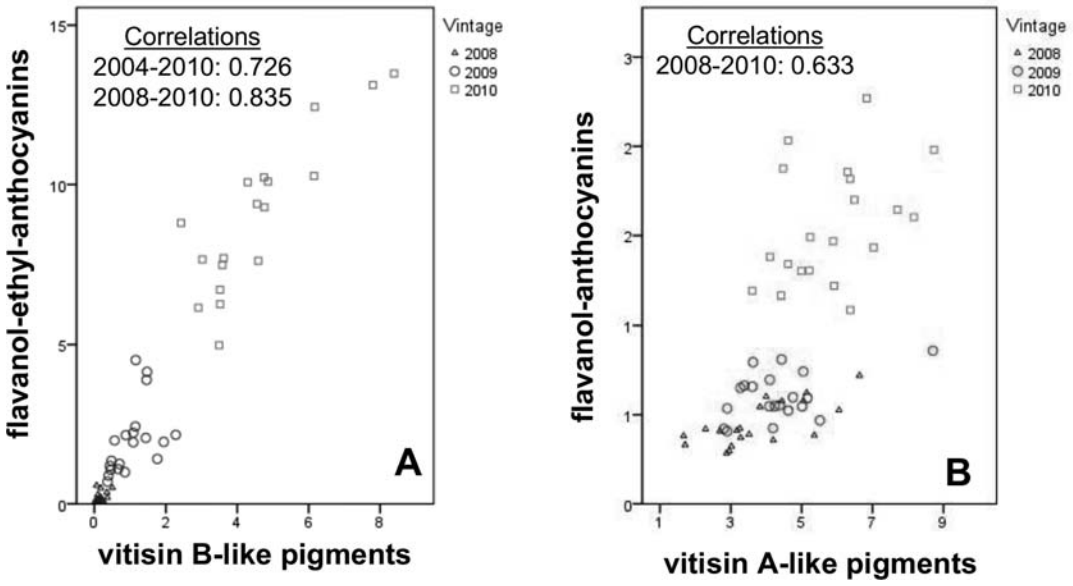


**Figure 5.** Plot A shows the correlation of the concentration sums between ethyl-linked flavanol−anthocyanins and vitisin B-like pigments and plot B the correlation between direct-linked flavanol−anthocyanins and vitisin A-like pigments.

after one year in the bottle and the concentration showed a tendency to stabilize after a few years.

With regard to the concentration of the flavanol−anthocyanins dimers, the ethyl-linked derivatives showed less stability than direct-linked flavanol−anthocyanic dimers (Figure 4). Fulcrand et al. suggested that acetaldehyde-induced pigment products, such as ethyl-linked flavanol−anthocyanin derivatives, are less stable than direct-linked flavanol−anthocyanic dimers.[29] The same research group also demonstrated the influence of pH on the concentration of these compounds,[18] but this should not apply in our case, because the pH values were similar and typical for the variety (Supporting Information, Table S1). Because vitisin B-like pigments are also considered in the literature as products of the reaction between anthocyanins and acetaldehyde,[8] we wanted to see if there is any correlation between them. The results obtained from comparison of the total amount of vitisin B-like pigments with ethyl-linked flavanol−anthocyanins showed a very good linear correlation between the 115 wines analyzed ($r$ = 0.726). The 115 samples derived from 7 different vintages and 20 independent wineries (55 commercial samples from 19 wineries and 60 experimental wines from a single winery). With a degree of freedom (df) of 113, such a value of the Pearson correlation coefficient should be considered surprisingly high (level of significance ≫ 0.001). The two-tailed significance level at 0.1% for df = 100 requires a value of $r$ = 0.32.

When restricted to semi-industrial wines only (3 vintages, 20 vineyards, 1 winery), the $r$ value for the same correlation was 0.835 (Figure 5A). Again, a level of significance ≫0.001 is required. The two-tailed significance level at 0.1% for df = 50 requires a value of $r$ = 0.45.

There are probably few situations in nature in which two variables are totally correlated, either because (a) one is the sole (or major) cause of the other or (b) both arise from a common sole cause.[31]

With regard to the hypotheses in the literature, based on wine model solution experiments, it could easily be assumed that this correlation is due to the fact that acetaldehyde is required for the formation of both vitisin B-like pigments and ethyl-linked flavanol−anthocyanins.[8,13,18] However, this approach has a major weakness in fully explaining the phenomenon. Because vitisin B-like pigments are more stable pigments than ethyl-linked flavanol−anthocyanins, if the two reactions are not connected but have such a similar selectivity/behavior, they should not be thermodynamically controlled. If they are kinetically controlled, we should not exclude a stronger relationship between the two products. Furthermore, the fact that wine is a far more complex matrix than model wine solution should not be underestimated; therefore, it is always risky to apply conclusions made in model solutions to wine, especially bearing in mind the variability in similar experiments from one wine to another. Recently, Arapitsas et al., on the basis of an untargeted study of wine microoxygentation, surmised that vitisin B-like pigments could be intermediate compounds for the formation of other pigments.[32] De Freitas et al. have also suggested that vitisin A-like pigments may work as intermediates for the formation of portisins in red Port wines.[8] Marquez et al. detected the formation of vitisins and anthocyanin−flavanol adducts (pigments according to our experiment correlated) formed in grapes during drying.The same authors tried to explain this through enzymatic activity, which may lead to production of both acetaldehyde and piruvic acid in dried grapes.[33] However, the issue of formation of these compounds in grapes calls for further explanation about where and how these compounds can be formed. In view of our results, and in association with the above literature, we cannot rule out the alternative hypothesis that vitisin B-like pigments could be intermediates to ethyl-linked flavanol−anthocyanins, starting from simple anthocyanic glucosides.

Furthermore, we also noted a good linear correlation between vitisin A-like pigments and direct-linked flavanol−anthocyanins ($r$ = 0.633) (Figure 5B). Accordingly, it could also be surmised that vitisin A is an intermediate of catechin−malvidin 3-glucoside synthesis. In this case, no correlation has been found or could be assumed between flavanol−anthocyanins and pyruvic acid (needed for the formation of vitisin A type pigments). Lately, Rentzsch et al. found a less significant correlation, in terms of $r$ value, for connecting the

concentration of pinotin A-like pigments and cinnamic acids in wine, which is a widely accepted correlation.[34] In any events, further experiments are needed to confirm these hypotheses.

The findings regarding the values of the ratio between the analogous B-ring tri- and disubstituted anthocyanins (malvidin + petunidin + delphinidin)/(cyanidin + peonidin) are also worth noting. It is common knowledge, and so the literature contains a wealth of papers using the anthocyanic profile for pattern recognition between the grape varieties. Biosynthesis of anthocyanins in the *V. vinifera* species is genetically controlled and involves two pathways, which are cyanidin-based (3′H) and delphinidin-based (3′,5′H). The ratio between the sum of the two pathways is characteristic for each variety.[2] This characteristic ratio is inherited by the wine from the grapes, although higher variability occurs.[1,5] The Sangiovese variety is already known to have a high level of variability,[27] because the different clones found produce grapes with a genetic variation, but this variability is not influenced significantly by the winemaking processes.[1]

Indeed, as shown in Figure 6, in our experiment Sangiovese wines also had a wide variability in relation to anthocyanic

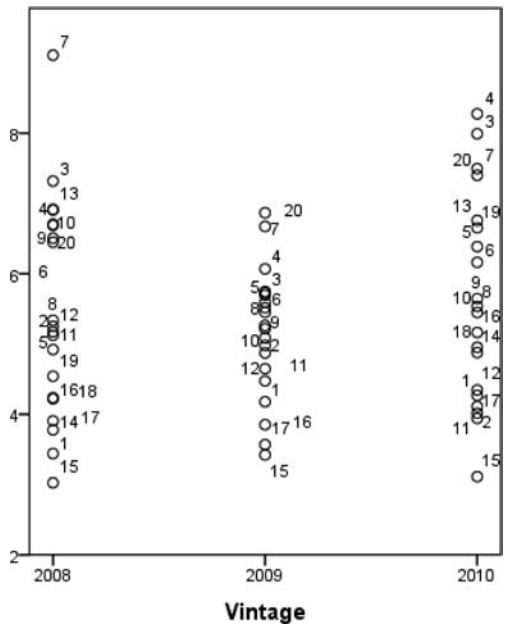


**Figure 6.** 3-Glucoside concentration ratio values of (malvidin + petunidin + delphinidin)/(cyanidin + peonidin) of the wines produced from grapes harvested from the 20 different vineyards over three consecutive years (2008−2010) on a semi-industrial scale. The numbers refer to the vineyard.

pattern, but this pattern was mainly dependent on the origin of the raw material. Therefore, wines from the grapes of the same vineyard have similar ratio values through the three vintages; low in all three years, like samples 15 and 17, or high, like samples 7 and 3 (Figure 6). The novelty, with regard to recognition of Sangiovese, came from the pigment pattern formed during aging. As shown in Figure 7, the pattern of the various anthocyanin derivatives known to form during wine aging could be genetically dependent and also have a strong correlation with the one of the grape anthocyanins. The two-tailed significance level at 0.1% for df = 50 requires a value of $r$ = 0.45, which means that all of the $r$ values reported in Figure 7 have a level of significance ≫0.001.

In more detail, whereas for vitisin B-like pigments this correlation would not seem to be affected by the various enological processes and/or the vintage (Figure 7A), vitisin A-like pigments and direct-linked flavanol−anthocyanins dimers were clearly different in the experimental and commercial wines (Figure 7B,C). This variation could be explained by the fact that the main factor distinguishing experimental from commercial wines is the lack of oak barrel aging, in association with the high influence of micro-oxygenation on the formation of these pigments occurring while the wine is kept in oak barrels. As a result of these observations, a series of questions arise. Do all grape anthocyanins (malvidin, peonidin, petunidin, cyanidin, and delphinidin) have the same reactivity in terms of forming aging pigments? Can one assume that for steric reasons ring B of the anthocyanins does not have any influence on the reaction between anthocyanins and acetaldehyde/pyruvic acid? According to the hypothesis, the ability of anthocyanins to act as an electrophile and participate in reactions of nucleophilic attack on the C4 of the C-ring should not be influenced by the electron resonant or steric effect of the flavylium B-ring. On the other hand, it is known that apart from pH, temperature, light, concentration, oxygen, the presences of metals, etc., the stability of the anthocyanins also depends on the B-ring structure.[7,35] More experiments are required in the future to explore the reactivity of the various anthocyanins, both in model solution and in different varieties of wines. Then again, if the profile of the pigments formed during aging follows a pattern depending more on genetic rather than technological reasons, this is of great importance for the traceability of wines. The significance of this characteristic is further extended in the case of wines such as Brunello di Montalcino, which have a long aging and market lifetime and in which the concentration of grape anthocyanins is hard to detect after a few years.

In addition, the possibility to follow with a single analysis the fate of such a large number of pigments, with different physicochemical features, could provide useful information concerning the color stability and protection of a wine, like Sangiovese, which has a delicate pigment profile. It is expected that detailed quality control covering all of the main wine pigments can provide superior information compared with measurement of the sole grape anthocyanins.

In conclusion, the detailed study of the Sangiovese pigment metabolites of 115 wines with a UHPLC-MS targeted method provided valuable results in relation to the behavior of the pigment profile during aging.

Because we analyzed large, representative samples of wines from seven different vintages, the behavior described in our graphs gives very general outcomes of how the profile of the pigments of Sangiovese wines will actually change through time. The likelihood that such a general observation would be true also for a specific wine is rather high, which is exactly why we preferred for this first experiment a deductive reasoning (following 115 different wines in search of general rules to be later further investigated) instead of the more widely adopted inductive reasoning (to follow a few wines or model solutions to demonstrate specific mechanisms to be later generalized).

A number of known issues in relation to the formation and evolution of main red wine pigments were also confirmed for Sangiovese. New findings emerged, due to the association of the concentration of grape-native anthocyanins and others formed during wine aging. Vitisin A-like pigments had a good correlation with ethyl-linked flavanol−anthocyanins, and direct-linked flavanol−anthocyanins showed a correlation with vitisin

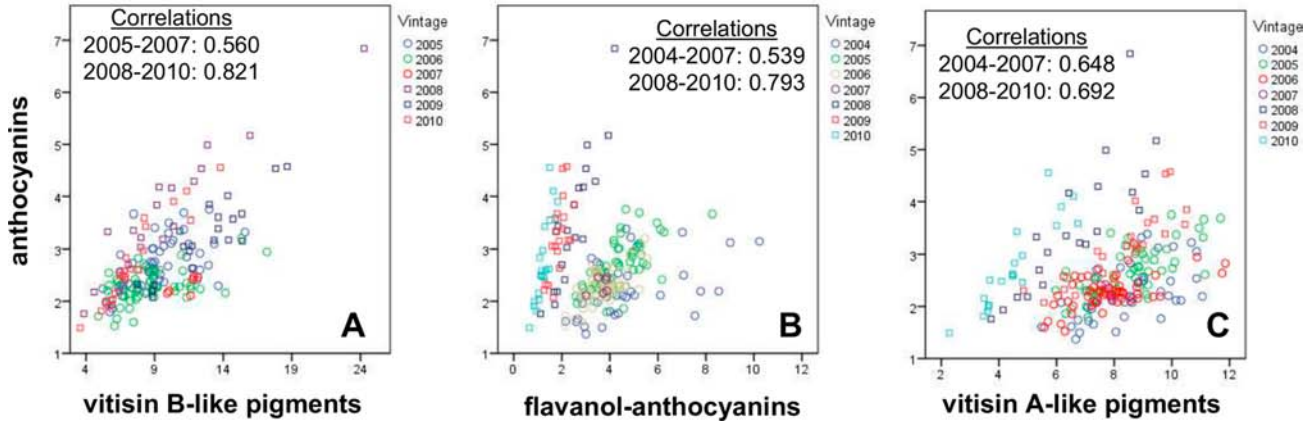


**Figure 7.** Correlation of the 3-glucoside concentration ratio values of (malvidin + petunidin + delphinidin)/(cyanidin + peonidin) with the concentration ratio values of their corresponding vitisin B-like pigments (plot A), direct-linked flavanol–anthocyanins (plot B), and vitisin A-like pigments (plot C).

A-like pigments. Furthermore, it was discovered that the formation of the pigments in wine follows a pattern that is variety dependent, at least for Sangiovese. All of this information allowed a better understanding of the complete Sangiovese wine pigment profile, which is considered useful to improve the color stability of the wine. It also provides a basis for understanding how grape pigments are transformed during winemaking and aging, essential knowledge for tracing the Sangiovese variety in wine. All of these results were made possible using modern instrumentation and the development of a carefully defined analytical protocol, which allows quantification of pigments with concentrations up to 2 orders of magnitude lower as compared to those accessible using conventional HPLC-DAD techniques, in a single run.

## ■ ASSOCIATED CONTENT

### Supporting Information

Vinification data and basic enological data and information about the grapes, alcoholic fermentation, malolactic fermentation, and the bottled wines. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

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