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ARTICLE

# Optimization of a Method for the Extraction and Quantification of Carotenoids and Chlorophylls during Ripening in Grape Berries (*Vitis vinifera* cv. Merlot)

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An extraction method to identify and quantify the carotenoid and chlorophyll profile of lyophilized tissue from unripe (green) to ripe (red) Merlot grape berries was developed. The RP-HPLC method baseline separated all of the carotenoids and chlorophylls and their derivatives. Problems encountered during sample storage and extraction are discussed as well as possible alternative methods. This study confirmed that carotenoids and chlorophylls decreased on a per berry ($\mu$g/berry) and concentration ($\mu$g/g) basis from veraison to harvest over two growing seasons. The carotenoid 5,8-epoxy-$\beta$-carotene was quantified for the first time in grapes and represents a significant amount of the total carotenoids present at harvest. All the carotenoids and chlorophylls except $\beta$-carotene appeared to be sensitive to seasonal variation in climatic conditions. Lutein and $\beta$-carotene were found to be the most abundant carotenoids present in Merlot grape berries together with chlorophyll a for both seasons studied.

**KEYWORDS: Chlorophyll; carotenoid; grape berry; HPLC; *Vitis vinifera*; Merlot**

## INTRODUCTION

Carotenoids have two main functions in the photosynthetic pathway of higher plants: photoprotection and light-harvesting. Photoprotection is the channeling of photochemical energy away from chlorophyll, whereas light-harvesting is the collection and subsequent transfer of light to chlorophyll via photochemical transduction (*1*). These functions are crucial for plant survival since excited triplet molecules can damage the photosynthetic apparatus and thus require both the effective transduction of light energy and dissipation of excess photochemical energy.

Carotenoids belong to the group of red or yellow pigments which absorb light between 450−570 nm in the visible light range (*2*). In natural sources, carotenoids occur mainly in the all-trans (*all-E*) configuration (*3*). Isomerization of *trans*-carotenoids to cis-isomers (*all-Z*) is promoted by contact with acids, heat treatment, and exposure to light (*2*, *4*). These alterations can have profound effects on the configuration and structure of these lipophilic pigments. The most common carotenoids present in mature (ripe) grapes are $\beta$-carotene and lutein, representing almost 85% of the total carotenoid content. They are accompanied by minor xanthophylls such as neoxanthin, violaxanthin, lutein-5,6-epoxide, zeaxanthin, neochrome, flavoxanthin, and luteoxanthin which make up the remaining proportion of total carotenoids (*5*−*11*).

The unique role of chlorophyll in photosynthetic light-harvesting and energy transduction in higher plants is well-known and documented in the literature (*12*). The structure of chlorophyll is a cyclic tetrapyrrole with a structure similar to the heme group of globins (hemoglobin and myglobin) and cytochromes. Chloropigments are susceptible to degradation either chemically or enzymatically. Enzymes, weak acids, oxygen, light, and heat can lead to the formation of a large number of degradation products (*12*). Although several types of chlorophyll exist, chlorophyll a is the major pigment in higher plants, and chlorophyll b is an accessory pigment. Chlorophyll a and chlorophyll b exist in a ratio of approximately 3:1 in higher plants (*12*).

Extensive research has been done on the carotenoid and chlorophyll content of food products and plants. During these studies, different analysis techniques, solvents, and extraction methods were used (*8*, *13*). The analysis and study of carotenoids in grape berries are important for the wine and grape industry since they were found to be precursors of important aroma compounds ($C_{13}$-norisoprenoids) present in wine. Furthermore, carotenoids and chlorophylls are also potential indicators of berry ripeness (*5*). However, a method to evaluate both the carotenoid and chlorophyll profiles of lyophilized grape tissue was not readily available at the outset of the current study.

An existing method for HPLC analysis of carotenoids in *Arabidopsis thaliana* leaf tissue (*13*) together with a combination of the extraction methods used by Oliveira et al. (*10*) and Mendes-Pinto et al. (*7*) were optimized for the simultaneous analysis of carotenoids and chlorophylls in green and red lyophilized berry tissue. Additionally, suggestions for further optimization and pitfalls of this method are discussed.

## MATERIALS AND METHODS

**Plant Material and Growth Conditions.** The plant materials used in this study were grape berries sourced from a nine-year-old commercial

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*Vitis vinifera* L. cv. Merlot vineyard (clone MO 9 clone grafted on Richter 110 rootstock) located in the Stellenbosch wine region, South Africa. Berries were harvested at different stages of ripening. Green berry tissue represents berries collected preveraison, and red berry tissue represents grapes at harvest (23 to 24 °Brix).

**Analytical Materials.** The following solvents were purchased from Sigma-Aldrich (Steinheim, Germany): methyl tertiary butyl ether (MTBE), ethyl acetate, diethyl ether, methanol, hexane, triethylamine, and 2, 6-di-*tert*-butyl-4-methylphenol (BHT). All of the chemicals used were of HPLC grade with the exception of sodium chloride (Fluka Chemie) and Tris base (Roche Diagnostics, Manheim, Germany) which were of analytical grade. The commercial standards $\beta$-apo-caroten-8-al (purity ≥96%), zeaxanthin (purity ≥96%), $\beta$-carotene (purity ≥95%), violaxanthin (purity ≥90.5%), neoxanthin (purity ≥88%), antheraxanthin (purity ≥88.4%), and lutein (purity ≥94%) were obtained from CaroteNature (Lupsingen, Switzerland). Chlorophyll a (purity ≥96%) and chlorophyll b (purity ≥94%) were acquired from Sigma-Aldrich (Steinheim, Germany). All of the ratios and percentages of solvents are indicated as volume per volume (v/v), unless otherwise stated.

**Preparation of Standards.** The commercial standards (violaxanthin, neoxanthin, lutein, zeaxanthin, chlorophyll a, and chlorophyll b; 100 $\mu$g/mL) were dissolved in chloroform, except for $\beta$-carotene (100 $\mu$g/mL), which was dissolved in chloroform/hexane (1:9) and $\beta$-apo-caroten-8-al (100 $\mu$g/mL) in ethyl acetate/methanol (1:4), respectively, with the addition of 0.1% (w/v) BHT. The stock solutions were divided in 1 mL aliquots into small amber high performance liquid chromatography (HPLC) vials and dried under a stream of nitrogen gas to prevent isomerization prior to storage at −80 °C. These standards were redissolved in the appropriate solvent prior to use. All dilutions were made in ethyl acetate/methanol (1:4) containing 0.1% (w/v) BHT. All dilutions were kept at −20 °C for no longer than 48 h and allowed to reach room temperature before analysis.

**Sample Preparation.** Grape berries were sampled at four different stages of ripening: preveraison (green), veraison, postveraison, and harvest (red). Four biological replicates of 25 berries each were randomly collected at each sampling date. Berries were immediately frozen after collection in liquid nitrogen to prevent any enzymatic or photodegradation. While berries were still frozen, their seeds were removed. The berry pericarps were ground under liquid nitrogen to a fine powder with an IKA A11 basic grinder (IKA-Werke GMBH & CO.KG, Staufen, Germany), where the tissue was lyophilized and kept at −80 °C prior to extraction and reverse phase (RP)-HPLC analysis. Sample preparation was done under subdued light at all times. For the determination of grape ripeness, an additional fresh 50-berry sample was collected from veraison onward and the pH of the expressed juice measured using a pH meter (Crison, Basic 20, Lasec Laboratory and Scientific Equipment Co). A 1:10 (w/v) extract of lyophilized tissue was extracted in 50% (v/v) ethanol in water and decolorized with polyvinylpolypyrrolidone (PVPP) (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany). The concentration of hexose sugars was determined on decolorized extracts using a commercial enzyme assay kit (R-Biopharm, Dramstadt, Germany).

**Extraction of Carotenoids and Chlorophylls from Grape Berries.** Extractions for RP-HPLC analyses were done on 100 mg of red and 50 mg of green berry tissue to which 500 $\mu$L of Millipore water and 10 $\mu$L of internal standard ($\beta$-apo-caroten-8-al 200 ng/$\mu$L) were added prior to extraction. In the final extraction protocol, the carotenoids and chlorophylls were extracted twice with 500 $\mu$L of diethyl ether/hexane (1:1). With each extraction, the sample was vortexed for 30 min in a 2 mL microcentrifuge tube after which it was centrifuged at 28 900*g* for 2 min. The upper organic phase of each extraction was collected, pooled, and dried under a stream of nitrogen. Dried samples were stored under a nitrogen atmosphere at −20 °C. Prior to RP-HPLC analysis, samples were dissolved in 200 $\mu$L of a 1:4 ethyl acetate−methanol solution containing 0.1% (w/v) BHT and centrifuged for 2 min at 28 900*g*. Samples were shielded from strong light and kept on ice during all procedures.

Different solvents and extraction times were investigated. Acetone and diethyl ether/hexane (1:1) were tested as possible extraction solvents comparing 5 and 30 min extraction times. The effect of exposure to normal laboratory light conditions (±32 000 lm) and low pH on carotenoid and chlorophyll degradation during the extraction procedure was also investigated. The standard extraction procedure using subdued light conditions was compared with normal laboratory light conditions. The effect of pH since green berry tissue had a lower pH than red berry tissue was evaluated by comparing the extraction procedure as mentioned above to an extraction procedure where the 500 $\mu$L of Millipore water added in the first step of extraction was replaced by 500 $\mu$L of a 50 mM Tris−HCl (pH 7.5) solution containing 1 M NaCl.

**Chromatographic Conditions.** The carotenoid and chlorophyll pigments were separated by RP-HPLC on an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a DAD system. An YMC30 column (250 mm × 4.6 mm) and YMC30 guard cartridge (10 mm × 4 mm, particle size 5 $\mu$m), both from YMC Europe (Schermbeck, Germany) were used. Chemstation software for LC3D (Rev.A.10.01[1635]; Hewlett-Packard, Waldborn, Germany) was used for data processing.

The RP-HPLC method of Taylor et al. (*13*) for the analysis of *Arabidopsis* leaves was evaluated for the analysis of carotenoids and chlorophylls in extracts from lyophilized grape berries. Selectivity was improved by using the YMC30 (250 × 4.6 mm; particle size 5 $\mu$m, separation factor $\alpha = 1.1$ for chlorophyll b and lutein) column for the more complex grape extract matrix compared to the YMC30 (100 mm × 2.1 mm; particle size 3 $\mu$m, separation factor $\alpha = 1.06$ for chlorophyll b and lutein) column used for the *Arabidopsis* leaf extracts. Mobile phase solvents consisting of 3% $H_2O$ (Millipore purification system, Millipore, Bellerica, MA, USA) in methanol containing 0.05 M ammonium acetate (solvent A) and 100% MTBE (solvent B) were used, where both solvents contained 0.1% (w/v) triethylamine. Various flow rates between 0.5 and 1.5 mL.min$^{-1}$ and gradient conditions were investigated. The optimal separation conditions were found to be a flow rate of 1 mL·min$^{-1}$ at 25 °C with an injection volume of 20 $\mu$L. Elution was done according to the following program: isocratic at 20% B for 20 min followed by a linear gradient from 20% B to 50% B in 4 min, isocratic at 50% B for 4 min followed by a linear increase to 68% B in 2 min, and isocratic at 68% B for 2 min followed by a linear decrease to 20% B. The column was equilibrated for 15 min at the starting conditions before each injection.

**Identification and Quantification of Carotenoids and Chlorophylls.** Identification of carotenoids and chlorophylls in Merlot grape samples was achieved by comparing retention times and visible spectra with commercial standards and published literature (*7*, *13*−*15*). The elution of the various carotenoid and chlorophyll pigments was followed at 420, 450, and 470 nm with a constant reference wavelength at 800 nm (*13*).

Standard curves for the quantification of carotenoids and chlorophylls were obtained by plotting amount (ng) against area which was obtained by triplicate injections. Chlorophyll a and b derivatives were quantified as chlorophyll a and b, respectively, while 5,8-epoxy-$\beta$-carotene and mutatoxanthin were quantified as zeaxanthin equivalents, and *cis*-violaxanthin, *cis*-neoxanthin and neochrome were quantified as neoxanthin equivalents. Liquid chromatography−mass spectrometry (LC-MS) was performed using a Waters API Q-TOF Ultima connected to a Waters UPLC (Waters Corporation, Massachusetts, USA) system. The same conditions used before were employed except that the mobile phases were slightly changed. Solvent A was 3% $H_2O$ in methanol, and solvent B was 100% MTBE. The chlorophylls and carotenoids were detected with an atmospheric pressure chemical ionization (APCI) system in the positive mode. A cone voltage of 35 V was used with the Q-TOF Ultima MS system. A capillary voltage of 3.5 kV with a desolvation temperature of 350 °C was also employed. The LC-MS analyses were performed to confirm the identification of chlorophyll and carotenoid derivatives for which commercial standards were not available.

**Limit of Detection and Quantification.** The limit of detection (LOD) was defined as the amount that results in a peak with a height three times that of the baseline noise. The limit of quantification (LOQ) was defined as the lowest injected amount which could be reproducibly quantified (RSD ≤ 5%).

**Selectivity, Precision, and Recovery.** The extraction method yield and selectivity were evaluated by doing mock extractions with mixtures of the commercial standards in quadruplicate. In a mock extraction, the extraction protocol is followed except that no grape tissue is present in the matrix. Recovery of individual carotenoids from the sample matrix was determined according to the amount extracted from the matrix spiked with known concentration of a mix of commercial standards minus the extract from the matrix alone. The normal extraction protocol was followed for all

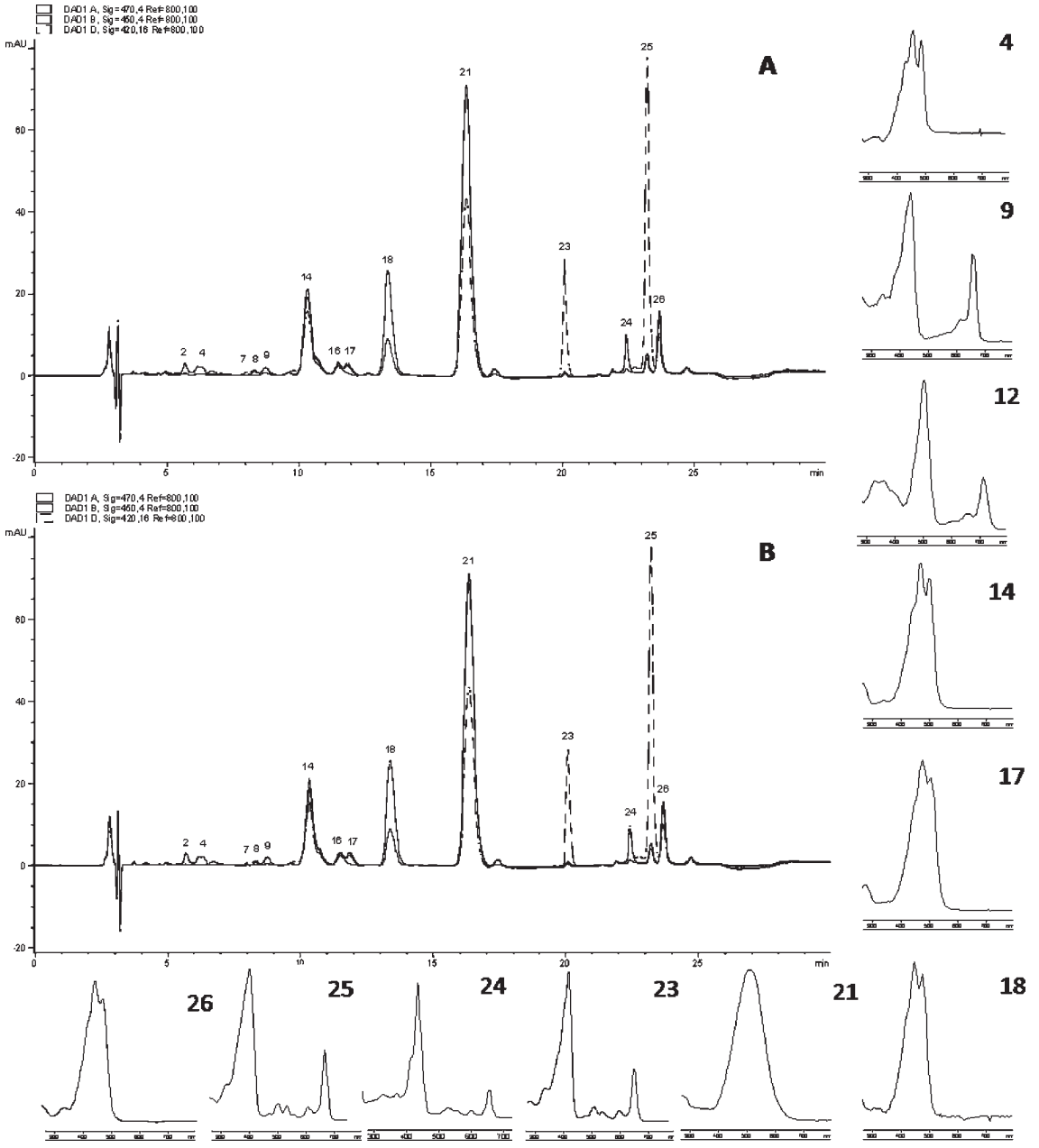


**Figure 1.** RP-HPLC profiles of the major carotenoids and chlorophylls in Merlot grape preveraison (**A**) and postveraison (**B**) berries: (1) *cis*-violaxanthin; (2) neochrome; (4) *cis*-neoxanthin; (7) luteoxanthin; (8) chlorophyllide a; (9) pyropheophorbide b; (12) chlorophyll b; (14) lutein; (16) mutatoxanthin; (17) zeaxanthin (18) 5,8-epoxy-$\beta$-carotene; (21) $\beta$-apo-caroten-8-al; (23) pyropheophytin b; (24) pheophytin b; (25) pheophytin a; (26) $\beta$-carotene; (27) 9-*cis*-$\beta$-carotene.

of the samples. Precision (repeatability) was similarly investigated at a low and high concentration in quadruplicate analyses.

## RESULTS AND DISCUSSION

**Identification and Quantification of Carotenoids and Chlorophylls in Grape Berries.** The following carotenoids and chlorophylls could be separated and identified by RP-HPLC by comparing UV−vis spectra and retention times of cv. Merlot grape sample peaks with those of commercial standards: neoxanthin, lutein, chlorophyll b, zeaxanthin, chlorophyll a, and $\beta$-carotene. In **Figure 1**, the HPLC chromatograms of red and green tissues are shown. It was observed that chlorophyll derivatives and degradation products, particularly in the green berry tissue, were present in fairly high amounts. These derivatives and degradation products were identified as chlorophyllide a, chlorophyll a′, pheophytin a, pyropheophorbide b, chlorophyll b′, pheophorbide b, pyropheophytin b, and pheophytin b in comparison with elution time and spectra according to the literature (*15*, *16*) and molecular masses obtained by MS (**Table 1**). Unknown carotenoid-like compounds were identified in comparison with the literature (*14*, *17*) and LC-MS as *cis*-violaxanthin, neochrome, *cis*-neoxanthin, luteoxanthin, flavoxanthin, auroxanthin, mutatoxanthin, *cis*-$\beta$-carotene, and 5,8-epoxy-$\beta$-carotene (**Table 1**). 5,8-Epoxy-$\beta$-carotene was identified according to its elution time, maximum absorbance, and fine structure in the methanol/MTBE mobile phase (*14*, *17*). *cis*-Neoxanthin was identified by the hypsochromic shift of 18 nm compared to all-*trans*-neoxanthin and the high intensity of the cis peak (*18*) (**Table 1**). Similarly, *cis*-violaxanthin was identified by the hypsochromic shift of 8 nm and the intensity of the cis peak (*18*). The cis-isomer of $\beta$-carotene was identified by comparison to the literature (*14*, *17*) retention time and taking into account

**Table 1.** Peak Identification of Grape Carotenoids and Chlorophylls on a C30 RP-HPLC Column

| | | | | absorbance | | | | | APCI-MS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| peak nr | compound | $t_R$(min) | $A_B$ | I | II | III | % (III/II) | % ($A_B$/II) | [M + H] | fragmentation ions (*m*/*z*) | identification |
| 1 | *cis*-violaxanthin | 5 | 320 | 410 | 430 | 458 | 44.7 | 17.0 | | | $t_R$, UV−vis |
| 2 | neochrome a | 5.7 | | 398 | 422 | 450 | 80.3 | | | nd | $t_R$, UV−vis |
| 3 | violaxanthin | 5.9 | | 418 | 438 | 470 | 83.4 | | 601.4 | 583.4 [M + H − 18] | MS, $t_R$, UV−vis |
| 4 | *cis*-neoxanthin | 6.2 | 314 | 398 | 418 | 442 | 66.0 | 19.0 | | nd | $t_R$, spectra |
| 5 | neoxanthin | 6.5 | | 412 | 436 | 464 | 93.9 | | 601.4 | 583.4 [M + H − 18] | MS, $t_R$, UV−vis |
| 6 | neochrome b | 6.9 | | 398 | 422 | 450 | | | | nd | $t_R$, UV−vis |
| 7 | luteoxanthin | 8.2 | | 400 | 422 | 441 | 100 | | 601.4 | nd | $t_R$, UV−vis |
| 8 | chlorophyllide a | 8.3 | | 430 | 658 | | | | 615.5 | nd | $t_R$, UV−vis |
| 9 | pyropheophorbide b | 8.4 | | 434 | 658 | | | | | | $t_R$, UV−vis |
| 10 | flavoxanthin | 8.9 | | 402 | 424 | 451 | 71.1 | | 585.9 | nd | $t_R$, UV−vis |
| 11 | neochrome | 9.3 | | 398 | 422 | 450 | 87.0 | | | | $t_R$, UV−vis |
| 12 | chlorophyll b | 9.6 | | 466 | 650 | | | | 907.5 | nd | $t_R$, UV−vis |
| 13 | auroxanthin | 10 | | 382 | 402 | 426 | 99.0 | | | | $t_R$, UV−vis |
| 14 | lutein | 10.3 | | 422 | 446 | 474 | 57.6 | | 569.0 | 551.4 [M + H − 18], 533.0 [M + H − 18−18] | MS, $t_R$, UV−vis |
| 15 | chlorophyll b′ | 11.1 | | 466 | 650 | | | | 907.5 | nd | |
| 16 | mutatoxanthin | 11.9 | 310 | 398 | 418 | 442 | 30.8 | 22.5 | 585.0 | 567.4 [M + H − 18], 549.4 [M + H − 18−18] | MS, $t_R$, UV−vis |
| 17 | zeaxanthin | 12.4 | | 422 | 450 | 478 | 22.4 | | 569.4 | 551.4 [M + H − 18] | MS, $t_R$, UV−vis |
| 18 | 5,8-epoxy-$\beta$-carotene | 13.2 | | 402 | 426 | 450 | 49.2 | | | nd | $t_R$, UV−vis |
| 19 | pheophorbide b | 14 | | | | | | | 607.4 | nd | $t_R$, UV−vis |
| 20 | chlorophyll a | 15.8 | | 430.8 | 666 | | | | 893.5 | nd | $t_R$, UV−vis |
| 21 | $\beta$-apo-caroten-8-al | 16.2 | | | 460 | | | | 417.0 | nd | MS, $t_R$, UV−vis |
| 22 | chlorophyll a′ | 17.5 | | 430.8 | 666 | | | | 893.5 | nd | nd |
| 23 | pyropheophytin b | 20.1 | | 418 | 662 | | | | 827.0 | nd | $t_R$, UV−vis |
| 24 | pheophytin b | 22.4 | | 434 | 654 | | | | 885.5 | nd | $t_R$, UV−vis |
| 25 | pheophytin a | 23.2 | | 410 | 666 | | | | 871.5 | nd | $t_R$, UV−vis |
| 26 | $\beta$-carotene | 23.7 | | 425 | 452 | 477 | 22.9 | | 537.4 | nd | $t_R$, UV−vis |
| 27 | 9-*cis*-$\beta$-carotene | 24.7 | 342 | 422 | 446 | 470 | 22.0 | 13.4 | 537.4 | nd | $t_R$, UV−vis |

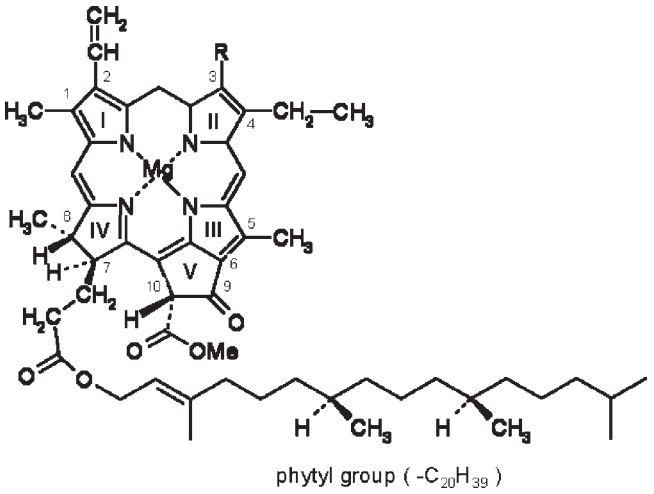


**Figure 2.** Structural formulas of chlorophyll a (R = Me) and b (R = CHO). In Pheophytins and pheophorbides, Mg is replaced by 2H.

the hypsochromic shift of 6 nm and increased intensity of the cis-peak (%$A_B/A_{II}$) (*18*) as well as by MS (**Table 1**). Mutatoxanthin was identified according to absorbance and retention time in similar mobile phase separations (*14*, *17*) and its molecular ion and fragment ions by MS (**Table 1**). Luteoxanthin and auroxanthin were identified according to their spectra and formation from violaxanthin when acidified with 0.1 M HCl (*19*). Violaxanthin has two 5,6-epoxide groups in its molecule, which can be transformed at low pH to luteoxanthin with one 5,6-epoxide and one 5,8-furanoid group. Finally, both of these gave rise to the isomer auroxanthin. Similarly, neoxanthin changed into mutatoxanthin with one 5,8-furanoid and then neochrome with two 5,8-furanoid groups under low pH conditions.

Other breakdown products were also observed, but in smaller quantities, including chlorophyllide a, chlorophyll a′, and pyropheophytin b (**Figure 2**). Chlorophyllides are formed when the phytyl group of the chlorophyll is cleaved. This is usually catalyzed enzymatically by the endogenous enzyme, chlorophyllase. Chlorophyll a′ is formed through epimerization of the C-10 center of chlorophyll a. Several studies have shown that heating causes isomerization of chlorophyll (*20*). Pyropheophytin is formed through decarbomethoxylation of the C-10 center of pheophytin (**Figure 2**).

The LOD and LOQ of the carotenoids and chlorophylls for which commercial standards were obtained were determined and are shown in **Table 2**. Repeatability was evaluated for low and high concentrations within the calibration range. The values obtained were within acceptable limits for this study (low concentration RSD $<4.5\%$ and high concentration RSD of $<3.5\%$).

**Extraction of Carotenoids and Chlorophylls from Grape Berries.** The selectivity of the RP-HPLC method and recovery of the extraction methods were evaluated. The recoveries of all commercial standards were $\geq 79\%$ from the mock extraction, which indicated that the extraction methodology was appropriate for the extraction of carotenoids and chlorophylls. The recovery of the commercial standards from red grape tissue was good ($\geq 77\%$), except for violaxanthin (49%) (**Table 3**), but improved to 63% when degradation products (*cis*-violaxanthin) were included. The recovery of chlorophyll a, chlorophyll b, violaxanthin, and neoxanthin, however, was very poor from green berry tissue. This result was found to be mainly due to the low pH of the tissue, which facilitates the degradation of chlorophyll a and b to pheophytin a and b, respectively. Violaxanthin and neoxanthin, respectively, degraded to auroxanthin and luteoxanthin and neochrome and mutatoxanthin. *cis*-Violaxanthin and *cis*-neoxanthin were also formed from violaxanthin and neoxanthin, respectively (*15*, *16*, *19*). cis−trans isomerization has been shown to be mainly mediated by heat (*19*). When the pheophytins a and b and pyropheophytin b forms were included in recovery calculations, recovery improved to 67%

for chlorophyll a and 113% for chlorophyll b. Poor recovery of both violaxanthin and neoxanthin from green tissue resulted in unreliable quantification of these compounds and were therefore not quantified in the Merlot grape berries. The green and red berry tissues were investigated because they represented the extreme stages of development in the different grape tissues analyzed. The differences in recovery between the green and red berry tissues were due to matrix differences, by which the pH differences between the tissue extracts would have made an important contribution to the recovery of pigments. There was significant variance in the recovery of compounds such as chlorophyll a and b and lutein, between the red and green grape tissue matrix. This extraction method was, however, used to obtain a profile of the carotenoid and chlorophyll pigments in grape tissues of different maturities and was not optimized for the extraction of a specific compound in a specific grape matrix.

**Table 2.** Limit of Detection and Quantification of Carotenoids and Chlorophylls As Determined by RP-HPLC

| standards | LOQ (mg/L) | LOD (mg/L) |
|---|---|---|
| β-apocaroten-8-al | 0.02 | 0.01 |
| antheraxanthin | 0.05 | 0.02 |
| β-carotene | 0.10 | 0.01 |
| zeaxanthin | 0.05 | 0.02 |
| violaxanthin | 0.02 | 0.01 |
| neoxanthin | 0.02 | 0.01 |
| lutein | 0.10 | 0.02 |
| chlorophyll a | 0.16 | 0.04 |
| chlorophyll b | 0.09 | 0.02 |

**Investigation of the Effect of Extraction Solvents, pH, and Light on Extraction Efficiency.** Acetone is a popular solvent used for the extraction of chlorophylls (*21*) and carotenoids (*22*) in leaves and various food types. Mendes-Pinto et al. (*8*) found that a mixture of hexane/diethyl ether 50/50 was the most effective for extracting both neoxanthin and β-carotene from grape berry tissue which are important aroma precursors in wine. These two solvents were evaluated as potential solvents for extracting both chlorophylls and carotenoids from grape tissue (**Tables 4** and **5**). Hemraj et al. (*21*) found that the amount of chlorophyll extracted is influenced by how finely the plant sample was ground and on the length of extraction time in the acetone. A longer extraction period facilitates a greater dissociation of the protein complex by acetone with removal of the chlorophyll pigments. Thus, an extraction time of 5 and 30 min was also investigated (**Tables 4** and **5**). This experiment was conducted as mock extractions with commercial standards.

Carotenoids and chlorophylls were found to be more stable in diethyl ether/hexane (1:1) than in acetone, which was reflected in the fact that fewer degradation products of carotenoids and chlorophylls were found when a 30 min extraction period was used. A 30 min extraction period also increased the extraction of more prevalent carotenoids without an increase in degradation products and was chosen as the optimal extraction time (**Table 4** and **5**).

The effect of pH and light (respectively) during the extraction method used in this study was evaluated by adding a buffer solution, which replaced the water in the extraction method (50 mM Tris-HCl, 7.5 pH, containing 1 M NaCl) (**Table 6**), and working under subdued light conditions instead of normal laboratory light conditions (data not shown).

**Table 3.** Recovery of Commercial Standards[a]

| compound | mock extraction% recovery[b,c] | mock extraction (ISTD) % recovery[d] | green tissue % recovery[e] | green tissue % recovery without breakdown products[f] | tissue % recovery[g] | red tissue % recovery without breakdown products[h] |
|---|---|---|---|---|---|---|
| violaxanthin | 89.0 | 101.0 | 0.0 | 0.0 | 63.2 | 49.1 |
| neoxanthin | 79.4 | 90.1 | 55.9 | 22.4 | 97.6 | 95.0 |
| chlorophyll b | 109.2 | 124.0 | 113.4 | 0.0 | 78.4 | 86.5 |
| lutein | 89.9 | 102.0 | 66.8 | 66.8 | 99.5 | 99.5 |
| zeaxanthin | 92.9 | 105.4 | 99.0 | 98.9 | 95.6 | 95.6 |
| chlorophyll a | 97.0 | 110.0 | 67.3 | 0.0 | 83.2 | 78.0 |
| β-apo-caroten-8-al (ISTD) | 85.1 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| β-carotene | 88.8 | 100.8 | 76.2 | 76.2 | 81.9 | 81.9 |

[a] All values are the average of 4 replicates. [b] Losses were compensated for according to internal standard (ISTD) in all cases, except in this column. [c] Mock extraction: on commercial standards without compensation according to ISTD. [d] Mock extraction: extraction of commercial standards. [e] Green tissue: extraction of commercial standards together with green lyophilized berry tissue. [f] Green tissue: recovery % includes all breakdown products: violaxanthin (sum of violaxanthin and *cis*-violaxanthin), neoxanthin (sum of neoxanthin and neochromes), chlorophyll b (sum of chlorophyll b, pheophytin b, and pyropheophytin b), lutein (lutein), zeaxanthin (zeaxanthin), chlorophyll a (sum of chlorophyll a and pheophytin a), and β-carotene (β-carotene) [g] Red tissue: extraction of commercial standards together with red lyophilized berry tissue. [h] Red tissue: recovery % includes all breakdown products: violaxanthin (sum of violaxanthin and *cis*-violaxanthin), neoxanthin (sum of neoxanthin and neochromes), chlorophyll b (sum of chlorophyll b, pheophytin b and pyropheophytin b), lutein (lutein), zeaxanthin (zeaxanthin), chlorophyll a (sum of chlorophyll a and pheophytin a), and β-carotene (β-carotene).

**Table 4.** Efficiency of Ethylether/Hexane (1:1) as Extraction Solvent for Carotenoids and Chlorophylls in Lyophilized Grape Tissue during Two Different Extraction Times[a]

| | ethylether/hexane | | | |
|---|---|---|---|---|
| | 30 min extraction | | 5 min extraction | |
| compound | amount recovered (ng) | % recovery | amount recovered (ng) | % recovery |
| violaxanthin | 58.62 ± 1.59 | 100.97 | 59.50 ± 1.03 | 102.48 |
| neoxanthin | 54.48 ± 1.72 | 95.61 | 55.17 ± 2.55 | 96.83 |
| antheraxanthin | 84.07 ± 2.01 | 100.39 | 84.87 ± 0.50 | 101.83 |
| chlorophyll b | 244.55 ± 6.62 | 101.45 | 247.44 ± 0.99 | 102.65 |
| lutein | 168.93 ± 4.67 | 102.70 | 168.61 ± 1.05 | 102.50 |
| zeaxanthin | 69.22 ± 2.93 | 102.40 | 68.83 ± 1.21 | 101.82 |
| chlorophyll a | 129.00 ± 4.90 | 102.58 | 129.58 ± 3.55 | 103.04 |
| β-carotene | 117.45 ± 1.90 | 122.89 | 114.56 ± 0.69 | 119.87 |

[a] Amounts recovered were calculated as the average of 3 replications.

**Table 5.** Efficiency of Acetone as Extraction Solvent for Carotenoids and Chlorophylls in Lyophilized Grape Tissue during Two Different Extraction Times[a]

| | acetone | | | |
|---|---|---|---|---|
| | 30 min extraction | | 5 min extraction | |
| compound | amount recovered (ng) | % recovery | amount recovered (ng) | % recovery |
| violaxanthin | 56.73 ± 0.46 | 97.72 | 55.45 ± 0.52 | 95.51 |
| neoxanthin | 53.62 ± 0.29 | 94.11 | 53.02 ± 0.38 | 93.06 |
| antheraxanthin | 81.79 ± 1.28 | 97.67 | 79.24 ± 1.03 | 94.62 |
| chlorophyll b | 235.81 ± 3.36 | 97.83 | 228.52 ± 4.98 | 94.80 |
| lutein | 163.55 ± 1.92 | 99.43 | 154.77 ± 2.47 | 94.09 |
| zeaxanthin | 62.73 ± 0.74 | 92.80 | 60.96 ± 1.08 | 90.17 |
| chlorophyll a | 125.78 ± 2.08 | 100.02 | 123.86 ± 0.55 | 98.49 |
| β-carotene | 119.37 ± 1.02 | 124.91 | 115.75 ± 1.60 | 121.12 |

[a] Amounts recovered were calculated as the average of 3 replications.

**Table 6.** Effect of pH on the Extraction of Carotenoids and Chlorophylls and the Formation of Degradation Products from Red and Green Berry Tissue[a]

| | percentage more pigments recovered in the presence of a buffer (pH 7.5) | |
|---|---|---|
| compound | red tissue | green tissue |
| neochromes | 0.0 | −23.0 |
| violaxanthin | 0.0 | 0.0 |
| neoxanthin | −17.3 | 100.0 |
| pyropheophytin b | −100 | −39.6 |
| chlorophyll b | 40.2 | 5.4 |
| lutein | −15.1 | −26.3 |
| zeaxanthin | 19.8 | −29.1 |
| chlorophyll a | 100.0 | 0.0 |
| pheophytin a | −184.2 | −25.7 |
| pheophytin b | −86.5 | −25.1 |
| β-carotene | −23.7 | −26.7 |

[a] All values were calculated from the average of 4 replicate analyses.

More chlorophyll b and neoxanthin were recovered from green grape tissue in the presence of the buffer (**Table 6**), although lutein, zeaxanthin, and β-carotene were recovered in lower amounts. Moreover, in the red grape tissue, chlorophyll a, chlorophyll b, and zeaxanthin showed higher recovery, while lutein, β-carotene, and neoxanthin were recovered in lower amounts. The increase in recovery of chlorophyll a and b was concurrent with lower amounts of their degradation product extracted/formed. An approximately 30% increase in the extraction of lutein, zeaxanthin, and β-carotene was found in green tissue under subdued light conditions compared to that in normal laboratory light conditions during extraction from both green and red berry tissues (data not shown). Working under subdued light conditions is a common practice when working with carotenoids and chlorophylls and is suggested to prevent cis/trans isomerization and degradation (*2*).

It is evident that the low pH of the berries, especially green berries (pH < 3.15) compared to red berries (pH ≥ 3.5), facilitated the transition of chlorophyll a and b to pheophytin a and b. The pheophytins are formed when the central Mg atom of the chlorophyll is replaced with a hydrogen ion (*23*), especially in the presence of plant acids from the vacuoles of extracted plant material (*20*). The addition of salts during the grinding of tissue has been recommended to prevent the formation of pheophytins, especially in plants with acidic cytoplasm. However, Strain et al. (*24*) have found that the addition of neither $CaCO_3$ nor $MgCO_3$ could totally prevent the formation of pheophytins in the extraction of chlorophyll from acidic tissue. In green tissue, even when extracted in the presence of a buffer, all of the chlorophyll a was already converted to pheophytin a, which indicates that some degradation had already taken place, possibly during the lyophilization of tissue and/or during storage (**Table 6**). In the red berry tissue extracts, pheophytins were also present even when the tissue was protected against the pH effect during extraction (**Table 6**). The amounts present in red tissue were, however, much less compared to that of green tissue. It is evident that although the addition of a Tris buffer to the extraction solvent decreased the formation of pheophytins significantly, it also decreased the extraction of carotenoids. In the lyophilized green tissue, there was no chlorophyll a present, and the buffer could thus only influence the extraction of pheophytin a, not its formation. In the green berry tissue, only 25% less pheophytin a and b was formed with the addition of the buffer during extraction, while in the red berry tissue, 184 and 86% less pheophytin a and b were, respectively, formed. This indicates that the buffer was not strong enough to neutralize the acid in the green tissue.

Although the extraction method used in this study was similar to those used by other authors (*8*, *10*) for grape berries, it is clear that it should be further optimized for the extraction of both carotenoids and chlorophylls and to minimize the effect of pH during extraction. Razungles et al. (*11*) mentioned the addition of 3 g of magnesium hydroxyl carbonate to the homogenate (100 g) of mature grape berries and 6 g to preveraison grape berries for the extraction of carotenoids. Razungles et al. (*11*) did not identify or report any cis-isomers of carotenoids, nor include the evaluation of chlorophyll in grape berries. Another study reporting the use of a buffer during carotenoid extraction was reported by Dias et al. (*25*) on Portuguese fruits and vegetables. The addition of sodium, magnesium, or calcium carbonate (0.10 g per gram of sample) to neutralize acids in tissue samples when extracting carotenoids have been suggested to avoid cis/trans isomeration (*2*). It is interesting to note that changes in pH within the thylakoid membrane facilitate these typical biochemical conversions in the xanthophyll cycle (*26*).

**Sample Processing and Storage.** Lyophilization of plant tissue is a well-known practice to preserve plant tissue samples and has been used widely to preserve grape tissue samples for the evaluation of carotenoid content (*6*, *22*). Craft et al. (*27*) reported in his work that the hydrocarbon carotenoids (carotenes) showed some degradation and that xanthophylls increased when tissue was lyophilized, which might be due to the more efficient hydrolysis of xanthophyll esters. Degradation of carotenoids in vegetables during lyophilization was also reported by Park (*28*). We suggest that degradation of chlorophyll is also possible during lyophilization since the water is removed from the tissue, concentrating the acid in the matrix, which might facilitate chlorophyll degradation.

Van den Berg et al. (*2*) suggested in a review on the potential of improvement in the carotenoid levels in food that food samples be stored at −20 °C short-term and long-term at −70 °C. Craft et al. (*29*) reported that carotenoids in serum samples stored

**Table 7.** Determination of Carotenoids and Chlorophylls ($\mu$g/Berry Fresh Weight) and Their Derivatives in Grape Berries at Different Sugar Ripeness Stages for the 2007 and 2008 Ripening Seasons[a]

| | | | | average $\mu$g/berry fresh weight[b] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| maturation stage | date | weight per berry (g) | % humidity | Zea | Lut | ep-$\beta$-car | $\beta$-car | *cis*- $\beta$-car | tot car | Chl a | Chl b | tot chl | juice pH | hexose sugars (mg) |
| preveraison | 11-Jan-07 | 0.657 | 90.54 | 0.15a | 1.28a | 0.02a | 0.76a | 0.09a | 2.55a | 17.56a | 5.25a | 23.49a | nm | 16.80 |
| veraison | 26-Jan-07 | 1.125 | 88.60 | 0.17ab | 1.77bc | 0.04ab | 0.85bc | 0.11ac | 3.00ac | 19.37bc | 6.70bc | 26.07bc | 3.14 | 86.60 |
| postveraison | 8-Feb-07 | 1.294 | 81.49 | 0.10c | 1.69b | 0.19c | 0.78b | 0.08d | 2.99a | 16.61bc | 4.89b | 22.78c | 3.39 | 124.22 |
| harvest | 7-Mar-07 | 1.403 | 76.50 | nd | 1.51a | 0.24ab | 0.58a | 0.06ab | 2.54a | 10.76a | 3.72c | 14.75a | 3.54 | 142.32 |
| preveraison | 10-Jan-08 | 0.773 | 92.66 | 0.28abc | 1.53c | 0.15a | 1.01a | 0.14c | 3.48bc | 16.91bc | 6.87 | 24.25bc | nm | 9.47 |
| veraison | 31-Jan-08 | 1.268 | 77.55 | 0.12a | 3.21ab | 3.00a | 1.46ab | 0.19ab | 8.86ab | 25.04ab | 11.4bc | 37.52ab | 3.21 | 115.17 |
| postveraison | 21-Feb-08 | 1.339 | 75.59 | 0.12a | 3.40b | 5.97d | 1.38c | 0.17d | 12.40b | 21.62b | 10.39b | 33.54b | 3.45 | 125.08 |
| harvest | 3-Mar-08 | 1.422 | 78.18 | 0.04a | 1.73a | 1.63a | 0.63ab | 0.07a | 4.48ab | 9.74ab | 4.49ac | 14.33ab | 3.51 | 137.10 |

[a] Carotenoids: zeaxanthin (Zea); lutein (Lut); 5,8-epoxy-$\beta$-carotene (ep-$\beta$-car); $\beta$-carotene ($\beta$-car); *cis* $\beta$-carotene (*cis* $\beta$-car); total carotenoids (Total car) calculated as the sum of all detected carotenoid like compounds. Chlorophyll: chlorophyll a (Chl a); chlorophyll b (Chl b); total chlorophyll (tot chl) calculated as the sum of all chlorophylls and detected chlorophyll derivatives. Significant differences: indicated with abcd when not bearing the same letter, indicating significant difference with $p \leq 0.05$ between plots for specific maturation stage. [b] Average calculated from 4 biological replicates each analyzed in triplicate; nd = not detected; nm = not measured.

at −70 °C were stable for at least 2 years. Van den Berg et al. (*2*) also recommended that when samples are stored for long periods before analysis, it is necessary to store samples together with reference samples from which the carotenoid content is known to compensate for degradation losses and to identify breakdown products easily. We found that dried aliquots of standards, especially chlorophyll a and violaxanthin were unstable and began to degrade shortly after storage. For this reason, samples were not stored for longer than 48 h at −20 °C.

**Changes in Carotenoid and Chlorophyll Content during Grape Ripening.** The most common carotenoids that were found in Merlot grape extracts were $\beta$-carotene, 5,8-epoxy-$\beta$-carotene, and lutein, representing approximately 85% of the total amount of carotenoids, accompanied by minor carotenoids such as *cis*-neoxanthin, violaxanthin, zeaxanthin, neochrome, flavoxanthin, luteoxanthin, and *cis*-$\beta$-carotene (**Table 7**). Similar results were found by Baumes et al. (*5*). All of the above-mentioned carotenoids and chlorophylls except 5,8-epoxy-$\beta$-carotene were previously reported to be found in grapes (*5*−*7*, *9*−*11*, *30*). No literature on the carotenoid and chlorophyll content of Merlot grape berries could be found to date.

Carotenoids and chlorophylls in grape pericarp tissue were investigated for both the ripening seasons studied with reference to the possible effect of climatic variation between seasons. The rate of transition and progression of the ripeness stages was similar between the two seasons studied in terms of the increase in berry weight, juice pH and hexose sugars (**Table 7**). Regarding the carotenoid content for the seasons studied, the carotenoid zeaxanthin was present in very small amounts in grape berries and degraded as ripening progressed. Preveraison zeaxanthin was present in berries at levels of 0.15 and 0.3 $\mu$g/berry fresh weight (fw) in 2007 and 2008 respectively and decreased to negligible amounts at harvest (**Table 7**).

Lutein, a well-known carotenoid present in grape berries showed an increase in the first part of the ripening (preveraison to postveraison) season for both seasons studied. The final content of lutein at harvest was higher than the preveraison level for both the 2007 and 2008 seasons, indicating that synthesis may have continued during veraison. For the 2008 season, the maximum level of lutein attained was approximately twice that observed in 2007, although by harvest, the lutein content was not significantly different between seasons. Similar amounts of lutein in grape berries were found by De Pinho et al. (*6*). Most authors have found a decrease in the lutein content of berries from veraison to harvest (*9*, *11*), but little data exists for preveraison levels of this pigment. Work by Oliveira et al. (*9*) indicated that for eight cultivars studied, most showed a decline in lutein following veraison, while one, Touriga Franca, showed a postveraison peak in lutein followed by a decline to harvest. It is, therefore, possible that cultivar differences may exist in the timing of lutein synthesis and degradation.

Similar to lutein, $\beta$-carotene showed an increase in content from preveraison to veraison, but declined thereafter, which is in agreement with the findings of other authors (*9*, *11*). Although levels of $\beta$-carotene were similar at harvest between the two seasons studied, the content of this pigment was higher in 2008 from preveraison to postveraison than in 2007. The cis-isomer of $\beta$-carotene was present in approximately ten times smaller quantities per berry fresh weight at harvest than $\beta$-carotene. cis-Isomers of $\beta$-carotene have been reported in grapes previously, although it is still uncertain if it is an artifact of sample preparation and analysis (*8*). 5,8-Epoxy-$\beta$-carotene, an oxidation product of $\beta$-carotene, increased as ripening progressed in contrast to most of the other carotenoids and chlorophylls, which decreased from earlier in the season until harvest. 5,8-Epoxy-$\beta$-carotene has been detected previously by Mendes-Pinto et al. (*8*) as an unknown compound in grape extracts from cvs Tinta Barroca, Touriga Francesa, and Tinta Roriz but was not quantified.

$\beta$-Carotene and lutein were the most common carotenoids found in mature Merlot berries representing more than 80% of the total portion of carotenoids analyzed per berry fresh weight in the 2007 season, which is in agreement with other work (*5*, *9*, *11*). However, in the 2008 season $\beta$-carotene and lutein represented only approximately 50% of the total carotenoids at harvest due to the high contribution of 5,8-epoxy-$\beta$-carotene.

The total carotenoid level for the 2008 season (4.48 $\mu$g/berry) at harvest was approximately two times greater than that found for the 2007 season (2.54 $\mu$g/berry) (**Table 7**). This result was mainly due to the significant increase of 5,8-epoxy-$\beta$-carotene in the 2008 season but was reflected in the profiles of all carotenoids and derivatives studied, apart from zeaxanthin. Since climatic conditions significantly influence the carotenoid profile of grape berries (*9*, *22*), the climatic conditions of the two growing seasons studied were compared using weather station data for the site. The 2007 and 2008 growing seasons were similar in terms of average day and night temperatures at the preveraison and veraison developmental stages (**Table 8**). During the ripening, the postveraison, and harvest periods, both average day and night temperatures were higher in the 2008 season. In terms of the solar radiation incident on the vineyard and the average sunshine

**Table 8.** Mean Day and Night Climate Differences during Four Ripening Stages (Preveraison; Veraison; Postveraison; Harvest) for the 2006/2007 and 2007/2008 Ripening Seasons[a]

| ripening stage | date | average day temperature (°C) | average night temperature (°C) | relative humidity day (%) | relative humidity night (%) | solar radiation (KW/h/m$^2$) | average sunshine per hour (%) | rain (mm) |
|---|---|---|---|---|---|---|---|---|
| preveraison | 1−11 Jan 07 | 22.50ab | 16.14ab | 58.63ab | 82.43ab | 1.43a | 73a | 0.00a |
| | 1−11 Jan 08 | 22.38ab | 17.23ab | 55.30acd | 72.95c | 1.36ab | 68abc | 0.06a |
| veraison | 12−25 Jan 07 | 25.95c | 19.08c | 51.38ac | 75.15c | 1.35ab | 70ab | 0.00a |
| | 12−25 Jan 08 | 25.12 cd | 17.92bc | 54.34acd | 75.73c | 1.34ab | 72a | 0.01a |
| postveraison | 26 Jan−8 Feb 07 | 24.91ac | 17.37abc | 53.90ac | 79.23ac | 1.33ab | 70ab | 0.00a |
| | 26 Jan−8 Feb 08 | 26.06 cd | 17.90bc | 47.81c | 74.94c | 1.39a | 74a | 0.02a |
| harvest | 9 Feb−7 Mar 07 | 21.47b | 16.08a | 61.39bd | 81.20ab | 1.17bc | 60bc | 0.09a |
| | 9 Feb−7 Mar 08 | 23.52ad | 17.12ab | 63.56b | 84.85b | 1.06c | 58c | 0.07a |

[a] Significant differences are only valid for each season comparing the same ripeness stage; significant differences are indicated with abcd if not bearing the same letter, indicating significant difference with $p \leq 0.05$).

hours, relative humidity, and rainfall, both the 2007 and 2008 growing seasons were similar. It would be expected that a decrease in total carotenoids in the 2008 season would occur in response to the higher temperature during the harvest period, which may have favored carotenoid degradation, but the contrary was seen. Rodriguez-Amaya et al. (*4*) stated that warmer temperatures and greater exposure to sunlight increase carotenogenesis but may also promote carotenoid photodegradation. It was found from studies in Brazil that papayas, cherries, and mangoes of the same cultivars produced in hot regions contained distinctly higher carotenoid concentrations than those in temperate climates (*4*).

Chlorophyll a (sum of chlorophyll a and pheophytin a), was found to be the most abundant pigment present in Merlot grape berries throughout the ripening season. Maximum levels of chlorophyll a and b were found at veraison for both the 2007 and 2008 seasons, and this peak was greater in the latter season. However, significant decreases in both chlorophyll types could be observed from postveraison to harvest, resulting in a similar final content for both seasons studied (**Table 7**). The grape chlorophyll a content found in the current study was 60-fold that reported by Oliveira et al. (*10*) in berries of cv. Touriga Nacional. These large differences could possibly be explained by variation in cultivar and terroir, but is more likely to be due to the inclusion of chlorophyll a derivatives into the estimation of total chlorophyll a content in the current study. As has been discussed in previous sections of this article, this was performed in response to the finding that chlorophyll a is degraded by low pH conditions during extraction.

Chlorophyll b (sum of chlorophyll b, pheophytin b, and pyropheophytin b) was present in berries in the beginning of the season at values of 5.2 to 6.9 $\mu$g/berry and degraded to 3.7 to 4.5 $\mu$g/berry by harvest (**Table 7**). The chlorophyll a content was four times that of chlorophyll b measured preveraison per berry. At harvest, chlorophyll b was present at 20 to 30% of its original concentration. Chlorophyll a, however, was only reduced to 50% of the initial amount that was observed preveraison per berry fresh weight by harvest. This finding is in agreement with work by Giovanelli and Brenna (*30*) that studied chlorophyll during ripening of two red cultivars, Barbera and Nebbiolo, and found 14 to 20% of the initial preveraison concentration of chlorophyll at berry maturity.

**Conclusions.** The RP-HPLC method baseline separated all of the carotenoids and chlorophylls and their derivatives. Recovery of standards from mock extractions was high, indicating that the extraction procedure was acceptable. However, it is clear that when the extraction recovery of the standards were tested in the matrix of the grape tissue the situation is less promising due to the high acid content of grape tissue. Violaxanthin, neoxanthin, and the chlorophylls were especially sensitive to low pH conditions, which facilitated their degradation. The degradation products of these compounds under acidic conditions were identified as pheophytin a and b, chlorophillide a, pyropheophytin b, *cis*-violaxanthin, *cis*-neoxanthin, neochrome, mutatoxanthin, and luteoxanthin. There is a possibility that some degradation products were already present in the tissue after lyophilization due to the concentration of organic acids in the tissue following water removal. More work is needed to investigate the effect of lyophilization and storage on the composition of grape tissues of different maturity. The extraction method for grape berry tissue at different ripening stages should also be optimized further to effectively neutralize tissue acidity, without compromising the extraction of carotenoids significantly, in especially green berry tissue. The question as to whether cis-isomers and chlorophyll degradation products are naturally present in grape berries or are formed during sampling and processing remains unanswered in the current study. This study confirmed that in general, carotenoids and chlorophylls decrease on a per berry ($\mu$g/berry) and concentration ($\mu$g/g) basis from veraison to harvest. 5,8-Epoxy-$\beta$-carotene was quantified for the first time in grapes and represents a significant amount of total carotenoids at harvest. All of the carotenoids and chlorophylls appeared to be sensitive to seasonal variation in climatic conditions. Lutein and $\beta$-carotene were found to be the most abundant carotenoids present in Merlot grape berries together with chlorophyll a for both seasons studied. The values of these carotenoids also correlated well with previous research. However, chlorophyll a was found in much larger quantities in Merlot berries compared to that in previous research on other varieties. This is possibly because in this study the chlorophyll degradation products were included in the calculation of chlorophyll a.

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