

Isolation, characterisation and properties of
8,8-methylmethine flavan-3-ol-malvidin-3-
glucoside pigments found in red wines

by

David F. Lee

8/8/2008

Thesis submitted for the degree of
Doctor of Philosophy

University of Adelaide
School of Agriculture, Food and Wine

Abstract

This study concerns the isolation, characterisation and physio-chemical properties of 8,8-methylmethine-(epi)catechin-malvidin-3-glucoside compounds found in red wines. 8,8-Methylmethine-(epi)catechin-malvidin-3-glucoside compounds were isolated via chromatographic methods developed in this study. The compounds were characterised via nuclear magnetic resonance spectrometry which, with the aid of molecular modelling, afforded their possible 3-dimensional structures. Their physio-chemical properties including ionisation and hydration constants, colour parameters and chemical stabilities were determined. The formation of 8,8-methylmethine-flavan-3-ol-malvidin-3-glucoside compounds and other pigments in wines was also studied.

8,8-Methylmethine-(epi)catechin-malvidin-3-glucoside compounds were also synthesised by condensing malvidin-3-glucoside with (epi)catechin in the presence of acetaldehyde. Diastereomers of 8,8-methylmethine-(epi)catechin-malvidin-3-glucoside pigments were isolated from the reaction using size-exclusion liquid chromatography followed by cation-exchange liquid chromatography.

The structures of the four 8,8-methylmethine-catechin (and epicatechin)-malvidin-3-glucoside diastereomers were determined using mass spectrometry and one and two-dimensional nuclear magnetic resonance spectroscopy. It was found that for all four compounds, the methylmethine bridge occurs at the 8-positions of malvidin-3-glucoside and (epi)catechin and that the 3-dimensional structural differences between the diastereomers is the positioning of the (epi)catechin moiety with respect to the glucoside group. One diastereomer has the (epi)catechin on the same side, with respect to the malvidin entity whilst it is on the opposite side for the other diastereomer. The proposed structures also afforded the malvidin entity protection from nucleophilic attack via steric hindrance by the (epi)catechin moiety.

8,8-Methylmethine-(epi)catechin-malvidin-3-glucoside pigments have greater colour stability with regards to changes in pH and SO₂ bleaching compared to malvidin-3-glucoside providing evidence that little or no hydration in aqueous solutions is occurring for these compounds. Further evidence for little or no hydration occurring is the presence

of isosbestic points in the UV-vis spectra observed for the 8,8-methylmethine-(epi)catechin-malvidin-3-glucoside in the pH range 2 to 7. Although the 8,8-methylmethine-(epi)catechin-malvidin-3-glucoside pigments have greater colour stability to pH, SO₂ and oxidation, compared to malvidin-3-glucoside, they have lower temporal stabilities and under both aerobic and anaerobic conditions they have significantly higher degradation rate constants than malvidin-3-glucoside.

The ionisation constants of the 8,8-methylmethine-(epi)catechin-malvidin-3-glucoside compounds were determined using high voltage paper electrophoresis (HVPE) and UV-visible spectroscopy. The first ionisation constants (pK_{a1}) of the 8,8-methylmethine-(epi)catechin-malvidin-3-glucoside compounds were found to be higher than that of malvidin-3-glucoside whereas the second and third ionisation constants (pK_{a2} and pK_{a3}) were found to be lower. The correlation of the ionisation constants between HVPE and UV-visible spectroscopy supports the proposal that there is little or no occurrence of hydration for the 8,8-methylmethine-(epi)catechin-malvidin-3-glucoside compounds in the pH range investigated.

8,8-Methylmethine-flavan-3-ol-malvidin-3-glucoside compounds were the major pigments formed during fermentations of chemically defined grape juice media containing malvidin-3-glucoside and various flavan-3-ols. The yeast strain used for fermentation had a major influence on the levels and rates of formation of these pigments during fermentation. The yeast strain used also has an important influence on wine pigment composition, concentration and evolution during maturation thereby affecting the colour density and hue of the resultant wines. The initial formation of 8,8-methylmethine-flavan-3-ol-malvidin-3-glucoside compounds and their subsequent gradual degradation during maturation, allowed a pool of malvidin-3-glucoside to be available for the formation of other colour stable and more temporally stable pigments.

Acknowledgements

Thanks to my principle supervisor Associate Professor Graham P. Jones and my co-supervisor Dr Ewald Swinny for their helpful advice and guidance.

Dr Robert Asenstorfer is gratefully acknowledged for his technical input, especially regarding the determination of pK values by HVPE and spectroscopic methods.

The people of the AWRI are acknowledged for their help, especially Yoki Hayasaka and Gayle Baldock for performing the mass spectrometry.

Thanks to Dr Michelle Walker and Colin McBryde for their guidance with the model wine making.

Thanks to Philip Clements for performing the NMR spectroscopy.

The staff of Kathleen Lumley College are thanked for providing accommodation conducive to studying.

This project was funded by the Cooperative Research Centre for Viticulture.

Declaration

This thesis describes my research carried out in the School of Agriculture, Food and Wine, the University of Adelaide and contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge this thesis contains no material previously published or written by any other person except where due reference is made in the text.

I give my consent for a copy of this thesis, when deposited in the Library of the University of Adelaide, to be available for the loan and photocopying.

David Lee

Contents

| | |
|---|------|
| <u>ABSTRACT</u> | II |
| <u>ACKNOWLEDGEMENTS</u> | IV |
| <u>DECLARATION</u> | V |
| <u>CONTENTS</u> | VI |
| <u>LIST OF TABLES</u> | XV |
| <u>LIST OF FIGURES</u> | XVII |
| <u>LIST OF ABBREVIATIONS</u> | XXI |
| <u>LIST OF PUBLICATIONS ARISING FROM THIS THESIS</u> | XXII |
| <u>REFEREED JOURNALS</u> | XXII |
| <u>CHAPTER 1 - LITERATURE REVIEW</u> | 1 |
| <u>1.1 INTRODUCTION</u> | 1 |
| <u>1.2 ANTHOCYANINS</u> | 1 |
| <u>1.3 FLAVAN-3-OLS</u> | 4 |
| <u>1.4 CARBONYL COMPOUNDS</u> | 6 |
| <u>1.5 COMPOUNDS FORMED BY THE REACTION BETWEEN POLYPHENOLS AND CARBONYL COMPOUNDS</u> | 6 |
| <i>1.5.1 Reactions of acetaldehyde to form wine pigments</i> | 6 |
| 1.5.1.1 Methylmethine-flavan-3-ol-anthocyanin compounds | 7 |
| 1.5.1.2 Vinyl-linked anthocyanin and flavan-3-ol compounds | 9 |
| <i>1.5.2 Vitisins and related compounds</i> | 12 |
| <i>1.5.3 Occurrence of vitisin-type and ethyl and vinyl-linked pigments in red wines</i> | 12 |
| <u>1.6 ISOLATION OF GRAPE ANTHOCYANINS AND WINE PIGMENTS</u> | 12 |
| <u>1.7 OBJECTIVES</u> | 13 |
| <u>CHAPTER 2 - MATERIALS AND METHODS</u> | 14 |
| <u>2.1 ISOLATION OF MV3G</u> | 14 |
| <i>2.1.1 Grape skin extract preparation</i> | 14 |
| <i>2.1.2 Size-exclusion chromatography</i> | 14 |
| <i>2.1.3 Cation-exchange chromatography</i> | 15 |
| <u>2.2 SYNTHESIS OF 8,8-METHYLMETHINE PIGMENTS</u> | 15 |

| | |
|--|-----------|
| <u>2.2.1 ISOLATION OF 8,8-METHYLMETHINE PIGMENTS</u> | 16 |
| 2.2.1.1 Size exclusion chromatography..... | 16 |
| 2.2.1.2 Cation-exchange chromatography..... | 16 |
| <u>2.3 SYNTHESIS AND ISOLATION OF PROANTHOCYANIDIN DIMERS</u> | 17 |
| 2.3.1 <i>Isolation of proanthocyanidins B1 and B2</i> | 17 |
| 2.3.2 <i>Synthesis and isolation of proanthocyanidins B3 and B4</i> | 17 |
| <u>2.4 ANALYTICAL HPLC</u> | 18 |
| 2.4.1 <i>LiChrospher 100 RP18</i> | 19 |
| 2.4.2 <i>Platinum EPS C18 Rocket</i> | 19 |
| <u>2.5 MASS SPECTROMETRY</u> | 19 |
| <u>2.6 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY</u> | 20 |
| <u>2.7 MOLECULAR MODELLING</u> | 20 |
| <u>2.8 ESTIMATION OF IONISATION CONSTANTS USING HIGH VOLTAGE PAPER ELECTROPHORESIS (HVPE)</u> | 20 |
| 2.8.1 <i>Calculations</i> | 20 |
| 2.8.2 <i>Apparatus</i> | 21 |
| 2.8.3 <i>Method for the Estimation of Ionisation Constants</i> | 21 |
| <u>2.9 ESTIMATION OF HYDRATION AND IONISATION CONSTANTS USING UV-VISIBLE SPECTROPHOTOMETRY</u> | 22 |
| 2.9.1 <i>Calculations</i> | 22 |
| 2.9.2 <i>Apparatus</i> | 22 |
| 2.9.3 <i>Method for the Estimation of Ionisation Constants</i> | 22 |
| <u>2.10 UV-VISIBLE SPECTROSCOPY</u> | 23 |
| <u>2.11 ESTIMATION OF THE STABILITY OF 8,8-METHYLMETHINE-(EPI)CATECHIN-MV3G COMPOUNDS TO CHANGE IN PH</u> | 23 |
| <u>2.12 ESTIMATION OF THE STABILITY OF 8,8-METHYLMETHINE-(EPI)CATECHIN-MV3G COMPOUNDS TO SULFUR DIOXIDE</u> | 24 |
| <u>2.13 ESTIMATION OF THE STABILITY OF 8,8-METHYLMETHINE-(EPI)CATECHIN-MV3G COMPOUNDS TO OXIDATION</u> | 24 |
| <u>2.14 WINE FERMENTATIONS</u> | 26 |
| 2.14.1 <i>Fermentation media</i> | 26 |
| 2.14.2 <i>Reagents</i> | 26 |
| 2.14.3 <i>Fermentation conditions</i> | 26 |

| | |
|---|-----------|
| <u>CHAPTER 3 - ISOLATION OF ANTHOCYANIN MONOGLUCOSIDES AND 8,8-METHYLMETHINE-(EPI)CATECHIN-MV3G AND PIGMENTS</u> | 28 |
| <u>3.1 INTRODUCTION</u> | 28 |
| <u>3.2 RESULTS</u> | 30 |
| <u>3.2.1 Isolation of MV3G</u> | 30 |
| <u>3.2.1.1 Size-exclusion chromatography</u> | 30 |
| <u>3.2.1.2 Cation-exchange chromatography</u> | 32 |
| <u>3.3 SYNTHESIS OF 8,8-METHYLMETHINE-(EPI)CATECHIN-MV3G COMPOUNDS</u> | 34 |
| <u>3.3.1 Isolation of 8,8-methylmethine-(epi)catechin-MV3G compounds</u> | 34 |
| <u>3.3.1.1 Size-exclusion chromatography</u> | 34 |
| <u>3.3.1.2 Cation-exchange chromatography</u> | 35 |
| <u>3.4 DISCUSSION</u> | 37 |
| <u>CHAPTER 4 - CHARACTERISATION OF 8,8-METHYLMETHINE-(EPI)CATECHIN-MV3G PIGMENTS</u> | 41 |
| <u>4.1 INTRODUCTION</u> | 41 |
| <u>4.2 RESULTS</u> | 42 |
| <u>4.2.1 Mass Spectrometry</u> | 42 |
| <u>4.2.2 Nuclear magnetic resonance spectrometry</u> | 43 |
| <u>4.2.2.1 Solvent system</u> | 43 |
| <u>4.2.2.2 ¹H NMR</u> | 44 |
| <u>4.2.2.3 ¹³C NMR</u> | 45 |
| <u>4.2.2.4 Determination of the position of the methylmethine bridge</u> | 47 |
| <u>4.2.3 Molecular Modelling</u> | 48 |
| <u>4.3 DISCUSSION</u> | 53 |
| <u>CHAPTER 5 - STABILITY OF 8,8-METHYLMETHINE-(EPI)CATECHIN-MALVIDIN-3-GLUCOSE PIGMENTS</u> | 57 |
| <u>5.1 INTRODUCTION</u> | 57 |
| <u>5.2 RESULTS</u> | 58 |
| <u>5.2.1 Stability of 8,8-methylmethine-(epi)catechin-MV3G compounds to change in pH</u> | 58 |
| <u>5.2.2 Stability of 8,8-methylmethine-(epi)catechin-MV3G compounds to sulfur dioxide</u> | 61 |

| | |
|--|------------|
| 5.2.3 <i>Stability of MC1 and MC2 to oxidation</i> | 62 |
| <u>5.3 DISCUSSION</u> | 67 |
| <u>CHAPTER 6 - PH-DEPENDENT EQUILIBRIA OF 8,8-METHYLMETHINE-(EPI)CATECHIN-MV3G PIGMENTS</u> | 74 |
| <u>6.2 INTRODUCTION</u> | 74 |
| <u>6.2 RESULTS</u> | 75 |
| 6.2.1 <i>HVPE determination of ionisation constants</i> | 75 |
| 6.2.2 <i>UV-visible spectrophotometric determination of ionisation constants</i> | 78 |
| 6.2.3 <i>Determination of ionisation constants using spectra area and centre of mass</i> . 80 | |
| 6.2.3.1 <i>Distribution profiles</i> | 81 |
| <u>6.4 DISCUSSION</u> | 83 |
| <u>CHAPTER 7 - FERMENTATIONS INCORPORATING ADDED MV3G AND FLAVAN-3-OLS</u> | 88 |
| <u>7.1 INTRODUCTION</u> | 88 |
| <u>7.2 RESULTS</u> | 89 |
| 7.2.1 <i>Fermentation Details</i> | 89 |
| 7.2.2 <i>Influence of Yeast Strain on Wine Pigment Formation</i> | 90 |
| 7.2.3 <i>Influence of Yeast Strain on Wine Colour</i> | 95 |
| 7.2.4 <i>Influence of Flavan-3-ols on Wine Pigment Formation</i> | 99 |
| 7.2.5 <i>Influence of Flavan-3-ols on Wine Colour</i> | 104 |
| <u>7.3 DISCUSSION</u> | 110 |
| <u>CHAPTER 8 - SUMMARY</u> | 114 |
| <u>REFERENCES</u> | 122 |
| <u>APPENDIX 1</u> | 129 |
| <i>Estimation of Ionisation Constants Using High Voltage Paper Electrophoresis (HVPE)</i> | 129 |
| Calculations | 129 |
| <i>Estimation of Hydration and Ionisation Constants using UV-visible Spectrophotometry</i> | 131 |
| Calculations | 131 |
| <u>APPENDIX 2</u> | 135 |

GRAMS/32 PROGRAM..... 135

List of Tables

| | |
|--|----|
| <u>Table 2.1. Parameters used in HVPE measurements</u> | 21 |
| <u>Table 4.1. Dimensions and cubic volumes of the proposed structures</u> | 51 |
| <u>Table 4.2. Dipole moments of the proposed structures</u> | 52 |
| <u>Table 5.1. Colour densities of MV3G, MC1, MC2, ME1 and ME2 at pH 1.0 and pH 3.6.</u> | 59 |
| <u>Table 5.2. Glories' colour densities of MV3G, MC1, MC2, ME1 and ME2 at pH 1.0 and pH 3.6.</u> | 60 |
| <u>Table 5.3. Colour colour densities of MV3G, MC1, MC2, ME1 and ME2 before and after the addition of sulfur dioxide at pH 2.8 and pH 3.3.</u> | 61 |
| <u>Table 5.4. Glories' densities of MV3G, MC1, MC2, ME1 and ME2 before and after the addition of sulfur dioxide at pH 2.8 and pH 3.3.</u> | 62 |
| <u>Table 5.5. Rates of degradation and half-lives of MV3G, MC1 and MC2 in model wines under anaerobic conditions at 20⁰C.</u> | 63 |
| <u>Table 5.6. Rates of degradation and half-lives pigments at 20⁰C in model wines under aerobic conditions.</u> | 65 |
| <u>Table 6.1. Macroscopic pKa values for MC1, MC2, ME1 and ME2 as derived using HVPE.</u> | 75 |
| <u>Table 6.2. Estimated optimum pHs for the occurrences of ionisation states for MC1, MC2, ME1 and ME2.</u> | 78 |
| <u>Table 6.3. Macroscopic pKa values for MC1, MC2, ME1 and ME2 as derived using spectroscopic methods.</u> | 80 |
| <u>Table 6.4. pKa values obtained from spectra area and centre of mass curves for MV3G, MC1 and MC2.</u> | 81 |
| <u>Table 6.5. Average macroscopic pKa values for MC1, MC2, ME1 and ME2 as derived using HVPE and spectroscopic methods.</u> | 81 |
| <u>Table 7.1. Evolution of MV3G, MC1 and MC2 and other pigments during preliminary fermentation and maturation</u> | 89 |
| <u>Table 7.2. Constituents, yeast strain, duration, alcohol content and pH of wines resulting from fermentations carried out at 30⁰C.</u> | 90 |
| <u>Table 7.3. Concentration of MV3G (mg/L) for model wines formed from the fermentations involving the yeasts EC1118, Syrah and BDX.</u> | 91 |

| | |
|--|-----|
| <u>Table 7.4. Concentration of MC1 and MC2 (mg/L MV3G eq) for model wines formed from the fermentations involving the yeasts EC1118, Syrah and BDX.....</u> | 92 |
| <u>Table 7.5. Concentration of pyranoanthocyanins (mg/L MV3G eq) for model wines formed from the fermentations involving the yeasts EC1118, Syrah and BDX.</u> | 93 |
| <u>Table 7.6. Concentration of other pigments (mg/L MV3G eq) for model wines formed from the fermentations involving the yeasts EC1118, Syrah and BDX.....</u> | 94 |
| <u>Table 7.7. Concentration of total pigments (mg/L) for model for wines formed from the fermentations involving the yeasts EC1118, Syrah and BDX.</u> | 95 |
| <u>Table 7.8. Concentration of MV3G (mg/L) for model wines formed from the fermentations involving the flavan-3-ols catechin, proanthocyanidin B3, proanthocyanidin B4, proanthocyanidins B1 & B2 and no flavan-3-ols.</u> | 100 |
| <u>Table 7.9. Concentration of 8,8-methylmethine pigments (mg/L MV3G eq) for model wines formed from the fermentations involving catechin, proanthocyanidin B3, proanthocyanidin B4, proanthocyanidins B1 & B2 and no flavan-3-ols.</u> | 101 |
| <u>Table 7.10. Concentration of pyranoanthocyanins (mg/L MV3G eq) for model wines formed from the fermentations involving catechin, proanthocyanidin B3, proanthocyanidin B4, proanthocyanidins B1 & B2 and no flavan-3-ols.</u> | 102 |
| <u>Table 7.11. Concentration of other pigments (mg/L MV3G eq) for model wines formed from the fermentations involving the flavan-3-ols catechin, proanthocyanidin B3, proanthocyanidin B4 and proanthocyanidins B1 & B2 and no flavan-3-ols.....</u> | 103 |
| <u>Table 7.12. Concentration of total pigments (mg/L) for model wines formed from the fermentations involving the flavan-3-ols catechin, proanthocyanidin B3, proanthocyanidin B4 and proanthocyanidins B1 & B2 and no flavan-3-ols.....</u> | 104 |

List of Figures

| | |
|--|----|
| <u>Figure 1.1 Anthocyanidin-3-glucoside</u> | 2 |
| <u>Figure 1.2. pH dependent structures of MV3G. A flavylium cation, B quinonoidal base, C hemiketal, D chalcone, E quinonoidal anion, F quinonoidal dianion (Glc = glucose).</u> 3 | |
| <u>Figure 1.3. Species distributions profiles of MV3G as determined by a) Brouillard and Delaporte (1977) and b) Asenstorfer et al. (2003). (— - flavylium cation,</u> | 4 |
| <u>Figure 1.4. Monomeric (A, B), 4-8 linked dimeric (C to F) and 4-6 linked dimeric (G to J) flavn-3-ols.</u> | 5 |
| <u>Figure 1.5 Reaction mechanism for the formation of 8,8-methylmethine-catechin-MV3G (Timberlake and Bridle, 1976)</u> | 7 |
| <u>Figure 1.6 Proposed reaction mechanism for the formation of MV3G-4-vinyl-catechin (Francia-Aricha et al., 1997).</u> | 10 |
| <u>Figure 1.7 Reaction mechanism proposed for the formation of vitisin B</u> | 11 |
| <u>Figure 3.1. HPLC chromatograms of the crude grape extract recorded at (a) 280 nm and (b) 520 nm.</u> | 30 |
| <u>Figure 3.2. HPLC chromatograms of TSK HW-40(F) fraction (1) at (a) 280 nm and (b) 520 nm.</u> | 31 |
| <u>Figure 3.3. HPLC chromatograms of TSK HW-40(F) fraction (2) at (a) 280 nm and (b) 520 nm.</u> | 31 |
| <u>Figure 3.4. HPLC chromatograms of TSK HW-40(F) fraction (3) at (a) 280 nm and (b) 520 nm.</u> | 32 |
| <u>Figure 3.5. HPLC chromatograms of TSK HW-40(F) fraction (4), after the removal of acetone, at (a) 280 nm and (b) 520 nm.</u> | 32 |
| <u>Figure 3.6. Elution profile of TSK HW-40(F) fraction (2) on sulfoxyethylcellulose. (□) Colourless products; (●) MV3GACET; (x) MV3G; (▲) PT3G; (◆) DP3G.</u> | 33 |
| <u>Figure 3.7. HPLC chromatogram of the MV3G and (a) catechin and (b) epicatechin reaction mixture recorded at 280 nm.</u> | 34 |
| <u>Figure 3.8. Fractions and compound concentration of the LH-20 chromatography of the MV3G, catechin and acetaldehyde reaction mixture. (◆) MV3G; (■) MC1 and MC2; (▲) catechin.</u> | 35 |
| <u>Figure 3.9. Elution profile on sulfoxyethylcellulose of LH-20 fractions 3 to 10, of the MV3G and catechin reaction mixture. (■) MV3G; (▲) MC1; (◆) MC2</u> | 36 |
| <u>Figure 4.1. Mass spectra of MC1 obtained in the positive ion mode</u> | 42 |

| | |
|---|----|
| <u>Figure 4.2. Structure and numbering of MC1, MC2, ME1 and ME2.</u> | 43 |
| <u>Figure 4.3. Proton signal for MV3G H-6 (arrowed) of MC2 with (a) HCl and (b) DCl in the solvent.</u> | 44 |
| <u>Figure 4.4. HMBC (solid lines) and ROESY (dashed lines) correlations for MC1, MC2, ME1 and ME2.</u> | 48 |
| <u>Figure 4.5. Proposed 3-dimensional structures for (a) MC1, (b) MC2, (c) ME1 and (d) ME2. Oxygen atoms are shown as solid spheres.</u> | 49 |
| <u>Figure 4.6. Alternative proposed 3-dimensional structures for (a) MC1, (b) MC2, (c) ME1 and (d) ME2. Oxygen atoms are shown as solid spheres.</u> | 50 |
| <u>Figure 4.7. Coordinate system used in the molecular modelling of MC1, MC2, ME1 and ME2.</u> | 51 |
| <u>Figure 4.8. Minimal energy positioning of the methyl-group for the catechin carbonium ion.</u> | 54 |
| <u>Figure 4.9. Proposed routes of formation of the 8,8-methylmethine MV3G and (epi)catechin diastereomers.</u> | 54 |
| <u>Figure 5.1. UV-visible spectra of (a) MV3G, (b) MC1 and (c) MC2 from pH 1.0 to 7.0.</u> | 59 |
| <u>Figure 5.2. Evolution of (a) spectra area and (b) spectra centre of mass for MV3G (◆), MC1 (■) and MC2 (▲) in the pH range 1.0 to 7.0.</u> | 61 |
| <u>Figure 5.3. Solution composition for the degradation of (a) MV3G (◆), (b)MC1 (■) and (c) MC2 (▲) in model wines under anaerobic conditions at 20⁰C. Evolution of other pigments (Δ) is also included.</u> | 63 |
| <u>Figure 5.4. Change in (a) colour density and (b) hue of MV3G (◆), MC1 (■) and MC2 (▲) in model wines under anaerobic conditions at 20⁰C.</u> | 64 |
| <u>Figure 5.5. Solution composition for the degradation of (a) MV3G (◆), (b) MC1 (■) and (c) MC2 (▲) in model wines under aerobic conditions at 20⁰C. Evolution of other pigments (Δ) is also included.</u> | 65 |
| <u>Figure 5.6. Change in (a) colour densities and (b) hues of MV3G (◆), MC1 (■) and MC2 (▲) in model wines under aerobic conditions at 20⁰C.</u> | 66 |
| <u>Figure 5.7. Steric hindrance of the 4-position to bisulfite bleaching. The bisulfite molecule and the 4-position are in black.</u> | 70 |
| <u>Figure 5.8. Postulated bridge cleavage mechanism of MC1 and MC2.</u> | 71 |
| <u>Figure 5.9. Schematic representation of the degree of movement for catechin of (a) MC1 and (b) MC2 after cleavage of the methylmethine-linkage. Malvidin aglycone – black, catechin – white, glucoside – grey.</u> | 72 |

| | |
|---|----|
| Figure 6.1. Relative mobilities of (a) MC1, (b) MC2, (c) ME1 and (d) ME 2 in an oxalate buffer as a function of pH. Equation 1.1 has been fitted for the estimation of pKa values. The coefficients of determination (r^2) for the four curves are (a) 0.99785, (b) 0.99703, (c) 0.99891 and (d) 0.99948. | 76 |
| Figure 6.2. Relative mobilities of (a) MC1, (b) MC2, (c) ME1 and (d) ME2 in an oxalate/citrate buffer as a function of pH. Equation 1.1 has been fitted for the estimation of pKa values. The coefficients of determination (r^2) for the four curves are (a) 0.99901, (b) 0.99636, (c) 0.99470 and (d) 0.99746. | 77 |
| Figure 6.3. Absorbance of MC1 as a function of pH with Equation 1.2 fitted for the estimation of ionisation constants. The analytical wavelengths used were (a) 480 nm, (b) 500 nm, (c) 535 nm and (d) 620 nm. The coefficients of determination (r^2) for the four curves were (a) 0.99901, (b) 0.99636, (c) 0.99470 and (d) 0.99760. | 79 |
| Figure 6.4. Species distribution as a function of pH for (a) MC1, (b) MC2, (c) ME1 and (d) ME2 (ionisation constants from Table 6.5). (- flavylum ion, - quinonoidal base, - quinonoidal anion, - quinonoidal dianion)..... | 82 |
| Figure 6.5. Quinonoidal base forms of MC1, MC2, ME1 and ME2. Arrows indicate locations of deprotonation of the flavylum form..... | 84 |
| Figure 6.6. Quinonoidal anion forms of MC1, MC2, ME1 and ME2 where the first deprotonation has occurred at the 4'-position. Arrows indicate locations of the second deprotonation..... | 85 |
| Figure 6.7. Quinonoidal dianion forms of MC1, MC2, ME1 and ME2. Arrows indicate locations of the third deprotonation..... | 85 |
| Figure 6.8. Steric hindrance of the 2- and 4-positions of the MV3G moiety to hydration. The water molecule and the 2- and 4-positions of the MV3G moiety are in black..... | 86 |
| Figure 6.9. Possible self-association/dimerisation structures of (A) MC1/ME1 and (B) MC2/ME2. Malvidin aglycone – black, epi(catechin) – white, glucoside – grey..... | 87 |
| Figure 7.1. Spectra evolution of the wines formed from the fermentations involving (a) EC1118, (b) Syrah and (c) BDX. sof = start of fermentation, eof = end of fermentation..... | 96 |
| Figure 7.2. Colour density evolution for wines formed from the fermentations involving the yeasts (■) EC1118, (■) Syrah and (□) BDX..... | 97 |
| Figure 7.3. Changes in Glories' colour density with time for wines formed with the yeasts (■) EC1118, (■) Syrah and (□) BDX..... | 98 |

| | |
|---|-----|
| <u>Figure 7.4. Hue evolution for wines formed from the fermentations involving the yeasts (■) EC1118, (■) Syrah and (□) BDx.....</u> | 99 |
| <u>Figure 7.5. Spectral evolution of the wines formed from the fermentations involving (a) catechin, (b) proanthocyanidin B3, (c) proanthocyanidin B4, (d) proanthocyanidins B1 & B2 and (e) no flavan-3-ols. sof = start of fermentation, eof = end of fermentation.....</u> | 106 |
| <u>Figure 7.6. Colour density evolution for wines formed from the fermentations involving the flavan-3-ols (■) catechin, (■) proanthocyanidin B3, (□) proanthocyanidin B4, (□) proanthocyanidins B1 & B2 and (▣) no flavan-3-ols.</u> | 107 |
| <u>Figure 7.7. Glories' colour density evolution for wines formed from the fermentations involving the flavan-3-ols (■) catechin, (■) proanthocyanidin B3) (□) proanthocyanidin B4, (□) proanthocyanidins B1 & B2 and (▣) no flavan-3-ols.....</u> | 108 |
| <u>Figure 7.8. Hue evolution for wines formed from the fermentations involving the flavan-3-ols (■) catechin, (■) proanthocyanidin B3, (□) proanthocyanidin B4, (□) proanthocyanidins B1 & B2 and (▣) no flavan-3-ols.</u> | 109 |
| <u>Figure 8.1. Proposed 8,8-methylmethine pigment substituents. A - chroman-3,5,7-triol, B - pyrogallol derivative</u> | 118 |
| <u>Figure 8.2. Proposed route for the formation involving glycolaldehyde of vinyl-linked MV3G and catechin compounds.</u> | 120 |

List of Abbreviations

| | |
|----------|---|
| AM1 | Austin Model 1 |
| CDGJM | Chemically Defined Grape Juice Media |
| CY3G | Cyanidin-3-glucoside |
| DEPT | Distortionless Enhancement by Polarization Transfer |
| DP3G | Delphinidin-3-glucoside |
| DQCOSY | Double Quantum Correlated Spectroscopy |
| HCl | Hydrochloric Acid |
| HMBC | Heteronuclear Multiple Bond Correlation |
| HMQC | Heteronuclear Multiple Quantum Coherence |
| HSQC | Heteronuclear Single Quantum Coherence |
| HVPE | High Voltage Paper Electrophoresis |
| MC1 | 8,8-methylmethine-catechin-malvidin-3-glucoside diastereomer |
| MC2 | 8,8-methylmethine-catechin-malvidin-3-glucoside diastereomer |
| ME1 | 8,8-methylmethine-epicatechin-malvidin-3-glucoside diastereomer |
| ME2 | 8,8-methylmethine-epicatechin-malvidin-3-glucoside diastereomer |
| MOPAC | Molecular Orbital Package |
| MV3G | Malvidin-3-glucoside |
| MV3GACET | malvidin-3-(acetyl)glucoside |
| MV3GCOU | malvidin-3-(coumaryl)glucoside |
| NaCl | Sodium Chloride |
| NMR | Nuclear Magnetic Resonance |
| PE3G | Peonidin-3-glucoside |
| PT3G | Petunidin-3-glucoside |
| ROESY | Rotational Nuclear Overhauser Effect Spectroscopy |
| TFA | Trifluoroacetic Acid |
| TLC | Thin Layer Chromatography |

List of publications arising from this thesis

Refereed journals

Lee, D. F.; Swinny, E. E.; Jones, G. P. NMR identification of ethyl-linked anthocyanin-flavanol pigments formed in model wine ferments. *Tetrahedron Lett.* **2004**, *45*, 1671-1674.

Lee, D. F.; Swinny, E. E.; Asenstorfer, R. E.; Jones, G. P. Factors affecting the formation of red wine pigments. In *Red Wine Color: Revealing the Mysteries*; A. L. Waterhouse and J. A. Kennedy, Eds.; American Chemical society: Washington, **2004** pp 125-142.

Asenstorfer, R.E., Lee, D.F. and Jones, G.P. Influence of structure on the ionization constants of anthocyanins and anthocyanin-like wine pigments. *Anal. Chim. Acta* **2006** *563*, 10-14.