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**LC/MS method development
for the separation of anthocyanins and
anthocyanin-derived pigments in red wines**

A dissertation
submitted in partial fulfilment
of the requirements for the
Postgraduate Diploma
in Applied Science

at
Lincoln University

by
Alistair S. P. Frey

Lincoln University, New Zealand

2015

Declaration

This dissertation contains no material which has been accepted for the award of any other degree or diploma in any University, and contains no copy or paraphrase of material previously presented by another person, except where due reference is made in the text.

Alistair Frey

November 2015

Abstract of a dissertation submitted in partial fulfilment
of the requirements for the Postgraduate Diploma in Applied Science

**LC/MS method development for the separation of anthocyanins and
anthocyanin-derived pigments in red wines**

by Alistair S. P. Frey

LC/MS method development was undertaken to investigate the factors affecting the separation of anthocyanin-3-*O*-glucosides in fractionated wine samples. The type and concentration of acid used to lower the pH of the HPLC solvents was found to be the most critical factor, with formic acid producing far better separations than acetic acid. The choice of solvent gradient, column temperature and flow rate were also found to affect separation.

The methods thus developed were successfully applied to the separation (by HPLC) and identification (by PDA/UV-Vis and MS) of a number of anthocyanin-3-*O*-glucosides, anthocyanin-3-*O*-acylglycosides, pyranoanthocyanins and polymeric pigments in young Pinot Noir and Cabernet Sauvignon wines. The relative amounts and proportions of anthocyanin-3-*O*-glucosides were compared between young wines vinified from four varieties of grape (Cabernet Sauvignon, Malbec, Merlot and Syrah) sourced from the Marlborough region of New Zealand.

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Abbreviations

α	Separation factor
Cy-3-Glu	Cyanidin-3- <i>O</i> -glucoside
DI-3-Glu	Delphinidin-3- <i>O</i> -glucoside
ESI	Electrospray ionisation
HPLC	High performance liquid chromatography
k	Retention factor
LC	Liquid chromatography
min	Minute(s)
MS	Mass spectroscopy
Mv-3-Glu	Malvidin-3- <i>O</i> -glucoside
<i>m/z</i>	Mass/charge ratio
PDA	Photodiode array
Pn-3-Glu	Peonidin-3- <i>O</i> -glucoside
Pt-3-Glu	Petunidin-3- <i>O</i> -glucoside
R _s	Resolution
SIM	Single ion mode
t _R	Retention time
t _M	Void time
w _{1/2}	Peak width at half-height
w _b	Peak width at base

1 Introduction

1.1 Anthocyanins and their derivatives

Anthocyanins are responsible for the colour of young red wines, and their reaction products play a significant role in determining the colour of red wines during subsequent ageing (Kennedy 2010). The five main anthocyanins found in red grapes and wines are delphinidin, cyanidin, petunidin, peonidin and malvidin in their 3-*O*-glucoside forms (abbreviated in this work as Dl-3-Glu, Cy-3-Glu, Pt-3-Glu, Pn-3-Glu and Mv-3-Glu, respectively). The structures of the anthocyanin-3-*O*-glucosides and references to their characterisation from grape or wine extracts are shown in Table 1.

The anthocyanins are observed in wine in a number of equilibrium forms. From the perspective of wine colour it is the flavylium form which is most important. The cationic flavylium form features a positive charge formally located at the 1-oxygen to give a fully aromatic C ring. The resulting π -conjugation throughout the three rings results in a strong peak absorbance around 500 nm, perceived as the colour red. Once formed the anthocyanins exist in equilibrium between the flavylium form and a number of other forms. The flavylium form can undergo hydration and deprotonation at C-2 to form the hemiketal form. The hemiketal C ring is no longer aromatic and the sp^3 C-2 breaks conjugation between the C and B rings resulting in no visible absorbance (i.e. colourless). The hemiketal and flavylium forms are the most and second most abundant forms of anthocyanins in wines. Deprotonation of the flavylium form can result in the formation of the quinoidal base or anionic forms. The quinoidal forms feature the formal loss of aromaticity in both A and C rings although conjugation is maintained from the 7-O through to the B ring, and the resultant visible absorbance results in a blue colour. The quinoidal base and its anions only appear in significant amounts at higher pH values than normally found in wine.

Pyranoanthocyanins are formed by the slow reaction of anthocyanins with compounds containing a polarisable double bond (or tautomericly accessible double bond) such as acetaldehyde, pyruvate, acetoacetic acid and vinylphenols from vinification onwards (Scollary 2010). Pyranoanthocyanins are more stable than their anthocyanin precursors, and their presence in aged wines helps explain the persistence of red wine colour long after the parent anthocyanin concentrations fall to zero. The change in hue from purple-red to orange-red associated with aged wines is also consistent with pyranoanthocyanin formation, since they typically exhibit hypsochromic shifts in the UV-Vis spectrum compared to the precursor anthocyanins.

Ethyl-bridged dimers form from the reaction of a flavan-3-ol (such as catechin) with a molecule of electrophilic, protonated acetaldehyde followed by reaction with an anthocyanin hemiketal (Scollary 2010). Ethyl-bridged dimers are typically violet or purple in colour and they are of interest as they have higher molar absorptivity than the parent anthocyanin, and are less sensitive to changes in pH.

1.2 Anthocyanin extraction from wine

The general procedure for extracting anthocyanins from grapes is to extract the skins with acidified alcohol followed by concentration and sometimes a crude purification using size exclusion chromatography (e.g. Toyopearl HW-40). Final separation is usually achieved *via* reverse phase HPLC using a C₁₈ column and acidified, gradient elution using water and MeCN or MeOH. The extraction and separation of polyphenols from wine, grapes and other plants has been the subject of a number of comprehensive reviews (Merken 2000, Valls 2009, Lorrain 2013).

Extraction of anthocyanins from wine is complicated by the presence of polymeric pigments and other high molecular weight compounds, which form a broad baseline peak that interferes with the identification of monomeric polyphenols. Methods for fractionating monomeric and polymeric polyphenols in wine samples to help simplify subsequent analyses include chromatography through LH-20 (Haslam 1980, Kantz 1991, Gu 2003), Toyopearl (Sarni-Manchado 1999, Remy 2000, Atanasova 2002, Guadalupe 2006, Sun 2006), polyamide resins (Ricardo da Silva 1990, Monagas 2003), C₁₈ mini-columns (Sun 2006) or solid-phase extraction columns (Monagas 2003, Oszmianski 1988, Sun 1998, Pinelo 2006). These methods suffer from procedural complexity (LH-20, Toyopearl), the irreversible binding of polymeric material to the column (polyamides) or the need for dealcoholisation prior to analysis (C₁₈-based solid phase extraction).

Fractionating of wine samples using divinylbenzene-*N*-vinylpyrrolidone copolymer-supported solid phase extraction has been reported (Jeffery 2008). Elution of wine directly through a disposable Oasis OLB brand column results in the immobilisation of most phenolic compounds including all coloured anthocyanins and polymeric pigments, without the need for prior dealcoholisation. Drying followed by elution with acidified acetonitrile, acidified methanol and finally neat formic acid provides three fractions, in which anthocyanins and later-eluting pigments such as pyranoanthocyanins (in fraction #1) are separated from polymeric pigments (fractions #2 and #3). This convenient and fast method was applied in the present study to provide the samples used for LC/MS method development.

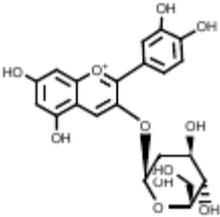
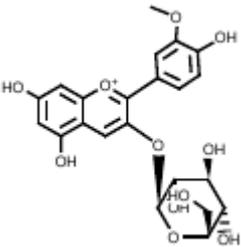
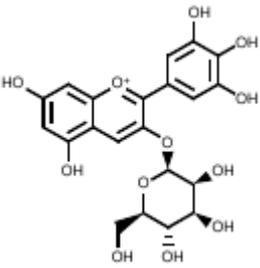
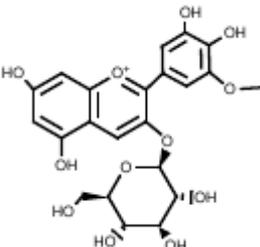
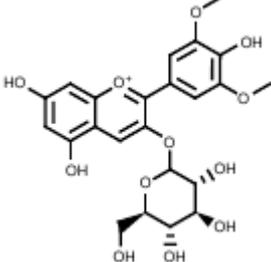
Anthocyanin structure	Name(s)	Characterisation references
	Cyanidin-3- <i>O</i> -glucoside	(Fernandes 2013, Fernandes 2010, Fernandes 2009, Yamakawa 1983, Krisa 1999, Mas 2000)
	Peonidin-3- <i>O</i> -glucoside	(Fernandes 2013, Krisa 1999, Mas 2000, Somers 1966, Yamakawa 1983)
	Delphinidin-3- <i>O</i> -glucoside Myrtillin	(Fernandes 2013, Fernandes 2010, Fernandes 2009, Mas 2000, Somers 1966, Garcia-Alonso 2004, Garcia-Estevez 2013)
	Petunidin-3- <i>O</i> -glucoside 1,6,7,8	(Fernandes 2013, Mas 2000, Somers 1966, Garcia-Alonso 2004)
	Malvidin-3- <i>O</i> -glucoside Oenin	(Fernandes 2013, Fernandes 2009, Mas 2000, Somers 1966, Garcia-Alonso 2004, Lopes 2007, Rivas-Gonzalo 1995, Santos 2014, Pissara 2004, Fulcrand 1996, He 2011, Hakansson 2003)

Table 1: Anthocyanin-3-*O*-glucosides found in *Vitis vinifera*, characterisation referencesObtained from Reaxys database, May 2015 (<http://www.reaxys.com>)

1.3 Anthocyanin LC/MS analysis

Detection of separated anthocyanins can be carried out using either a photodiode array (PDA) and/or electrospray mass spectrometric (ESI MS) detection at the output of the HPLC column (Dong, 2006). PDA detection of anthocyanins can be carried out across a UV-Vis range of ca. 200-700 nm. Subsequent analysis of absorbance around 520 nm allows coloured anthocyanins and related pigments to be discriminated even in the presence of co-eluting analytes which do not absorb in the middle of the visible region, such as flavanols. PDA detectors can also record full UV-Vis absorbance spectra which can be analysed post-run. ESI MS detection can run in with positive or negative ionisation modes, providing parent ion mass to charge (m/z) data. MS detectors featuring single ion mode (SIM) detection allow the simultaneous collection of MS data in a number of individual channels each set to a m/z ratio of interest. This provides the ability to individually detect and quantitate a range of compounds simultaneously (e.g. co-eluting from an HPLC column), a useful feature during LC/MS method development. The MS detector is placed downstream of the non-destructive PDA detector, allowing UV-Vis absorbance and MS data to be correlated.

The acidity of the solvents employed for HPLC separation of anthocyanins is particularly important since the different equilibrium anthocyanin forms have different column affinities, resulting in peak broadening. Furthermore, the quantitation of anthocyanins by 520 nm absorbance is dependant on the equilibrium concentration of the coloured flavylium form. For these reasons HPLC solvents are acidified to low pH values to minimise the impact of non-flavylium anthocyanin forms on the HPLC separation and UV-Vis detection. However, high acidity implies high H^+ concentrations, which can hamper analyte ionisation in the MS detector due to formation of ion pairs (Garcia-Beneytez 2003), so methods employing low pH to promote detection of anthocyanins might not be ideal for the detection of later-eluting compounds requiring maximum MS signal-to-noise ratios for positive identification.

A survey of a number of papers dealing with the quantitation of anthocyanins from wine, grape or other berry extracts includes examples of acidification using a variety of acid types and concentrations. Formic acid is most commonly used, at 10% (Favretto 2000, Kammerer 2004, Núñez 2004, Tian 2004, Monagas 2004, Ruberto 2006) or 1% (Seeram 2006), although trifluoroacetic acid also occasionally used, either alone (Garcia-Beneytez 2003) or in conjunction with another acid, e.g. acetic acid (Wang 2004). However, discussions of why certain acids are preferred to others are absent from the literature.

1.4 HPLC metrics

A few basic HPLC parameters need to be defined to allow the separating ability of a given LC/MS method to be numerically measured (Dong 2006, Bidlingmeyer 1992). The time taken for an analyte to elute through the column is the *retention time* t_R . The time taken for a molecule which has zero affinity for the column to pass through is the *void time* t_M . The *retention factor* (k) describes the ratio of these two: $k = (t_R - t_M) / t_M$, and is effectively the ratio of time spent in the stationary vs mobile phases.

The shape of individual peaks is also important in determining how effective a given separation is. Peak width can be measured either at the base (w_b) or at half-height ($w_{1/2}$). Measuring peak width at the base of a Gaussian curve is difficult since deciding where a peak finishes is subjective and prone to varying interpretation depending on the scale of the peak and the amount of noise in the baseline. Half-height width measurements avoid these issues since the peak height is usually unambiguous and the baseline can be determined by taking the mid-points (averaging out) of any noise present. Base width (for Gaussian shaped peaks) can be found from the half-height width: $w_b = 1.70(w_{1/2})$.

The two HPLC metrics which are of greatest utility in assessing in assessing the efficacy of a given LC/MS method are the *separation factor* (α) and *resolution* (R_S). The separation factor is a measure of how selective a given method is, and is found for any two peaks from the ratio of their respective retention factors: $\alpha = k_2 / k_1$. Resolution is the difference in retention times of two peaks divided by the average peak widths, giving a numerical measure of the degree of overlap or separation between two peaks with possibly different peak widths: $R_S = (t_{R2} - t_{R1}) / (0.5 w_{b2} + 0.5 w_{b1})$. As a rough guide, for two peaks to be considered baseline separated an R_S value of 1.5 - 2 is required. R_S is a better indicator of the utility of an HPLC separation since it accounts for peak widths, so it will be used in this study as the principal indicator of the effects of variations to LC/MS methods. In cases where peak widths cannot be estimated accurately (e.g. due to non-Gaussian peak shapes), α will be used as an alternative indicator.

1.5 Scope of study

An LC/MS method for the separation of polyphenols has been developed in the Harrison research group (Leblanc 2015). The method provided good separation of many flavanols from fractionated red wine samples (Jeffery 2008), however the five main anthocyanins co-eluted with almost no separation and non-Gaussian peak shapes. Although MS scanning ion mode (SIM) can identify and

quantitate overlapping peaks, an HPLC method providing baseline separation of the five anthocyanins was required, so that future anthocyanin analyses could be carried out using PDA detection alone.

The primary aim of this project was to develop an LC/MS method which produces baseline separation of the five anthocyanin-3-*O*-glucosides from a fractionated wine sample. Published methods for the HPLC quantitation of anthocyanins use a variety of acid types and concentrations, so we wished to systematically evaluate the effect of acid type, acid concentration, solvent gradient and other variables on the resolution of anthocyanins and their detection by PDA and MS.

A secondary aim of the project was to expand the optimised anthocyanin LC method(s) to the separation and identification "late-eluting pigments" taken to be anthocyanin-3-*O*-acylglycosides, pyranoanthocyanins and polymeric pigments. This would include an investigation of the potential trade-off between anthocyanin separation (promoted by low pH) and the MS detection of late-eluting pigments which are present at low concentrations in young wines (hampered by low pH).

The third aspect of the project involved applying the optimised anthocyanin and late-eluting LC/MS methods to fractionated samples of young wines (ca. ~4 months old) made from four different varieties (Cabernet Sauvignon, Malbec, Merlot and Syrah) obtained from the Church Road winery (Marlborough region, New Zealand). Time constraints precluded the incorporation calibration standards, so the quantitation of anthocyanins in the Church Road wine samples was to be evaluated only on a relative basis.

2 Methods and materials

2.1 Wine fractionation

Wine was fractionated according to the published method (Jeffery, 2008). An Oasis HLB column (3 mL, 60 mg, 30 μ m, plastic syringe type) was conditioned with 2 mL methanol followed by 2 mL water. The wine sample (1 mL) was added to the column as soon as the 2 mL water had eluted and the frit of the cartridge was still wet. All coloured compounds adhered to the column, which was dried by the passage of dry nitrogen gas (30 min). The first fraction was obtained by eluting a total of 40 mL 95% MeCN/5% 0.01 M HCl through the column. The second fraction was obtained by eluting 5 mL MeOH containing 0.1% formic acid, and the third fraction was obtained by passing adding 0.3 mL neat formic acid to the column followed by 2.7 mL 95% MeOH/5% water. The three

pink/purple coloured fractions were stripped to dryness under reduced pressure; the formic acid in the third fraction required a stream of dry nitrogen gas for complete removal. The highly coloured residues obtained from the first two fractions were redissolved in 1 mL 10% EtOH/0.1% formic acid for later analysis, whilst the third fraction was redissolved by adding 0.01 mL neat formic acid followed by approx. 0.05 mL 10% EtOH/0.1% formic acid to dissolve the sample, then ca. 1 mL 10% EtOH/ 0.1% formic acid. The solutions thus prepared were stored at -18 °C and warmed to room temperature shortly before analysis.

2.2 LCMS - Instrumentation

Liquid chromatography was performed on a Shimadzu 2010 instrument equipped with two binary LC-20AD pumps, a DGU-20A5 solvent degasser, SIL-20AC auto-sampler, CTO-20A column oven, SPDM-20A PDA detector, CBM-20A comms bus module, and a LCMS-2010EV mass spectrophotometer with ElectroSpray ionization (ESI) probe simultaneously operated with scanning and SIM modes, in both positive and negative ion modes. The mass spectrometer heat block temperature was 200 °C and the curved desolvation line (CDL) temperature was 200 °C. Both nebulising and drying gas were nitrogen, the nebulising gas flow at 1.4-1.6 L/min and drying gas pressure at 0.14 MPa. Detector voltage was 1.5 kV, interface voltage was 4.5 kV and CDL voltage was -45 V. MS data was collected in the range $m/z = 250 - 700$ amu. The PDA detector wavelength collected data from 200 nm to 700 nm and quantification was performed at 520 nm. The HPLC column was an Alltima C-18, 250 x 2.1 mm, particle size 5 μ m, (Alltech, USA).

2.3 LCMS - Method

All LC/MS runs used water as the polar solvent A and acetonitrile for solvent B. Both solvents A and B were acidified with the same type and concentration of acid. For all runs both PDA and MS detection were employed. The MS detector simultaneously collected scan data ($m/z = 250$ to 700 amu) and SIM data (single ion mode) in both negative and positive ion modes, with the SIM channels set to record the m/z values corresponding to 35 pigment compounds (five anthocyanin-3-*O*-glucosides, malvidin-3-*O*-galactoside, 11 anthocyanin-3-*O*-acylglycosides and 18 pyrano-anthocyanins and polymeric pigments) previously reported in a literature study of Cabernet Sauvignon (Wang 2004).

The void time (t_M) of the column was 3.07 min, corresponding to the retention time of the very first peak to appear on the PDA or MS output of any given run (due to trace amounts of highly polar

compounds unretained by the column). A similar value of 2.81 min was calculated by considering the void volume of the C_{18} column (0.563 cm^3 , assuming the pore volume is 0.65 times the empty column volume, Dong 2006) multiplied by the normal flow rate (0.2 mL/min), so the empirically derived value of 3.07 minutes was deemed reasonable and was used in calculating retention factors (k) and separation factors (α).

Pure, characterised anthocyanins were not used for this method development due to the very high cost of commercial samples (e.g. \$590 (ex GST) for 1 mg peonidin-3-*O*-glucoside chloride, Aldrich New Zealand, October 2015). Instead, purified wine samples containing a naturally occurring mixture of anthocyanins were used as a source of target analytes. Pinot Noir was considered an appropriate variety for LC/MS method development as the absence of anthocyanin acylglycoside derivatives in Pinot Noir grapes would provide simpler chromatograms than other grape varieties. A young sample obtained from the fermentation tank of Pinot Noir grapes sourced from Raupara, Marlborough during the 2015 vintage was chosen to provide high concentrations of anthocyanins. This wine sample was previously fractionated (Jeffery 2008) as part of an earlier study undertaken within the Harrison research group (Leblanc 2015). The first fraction was used for LC/MS development in this study either neat, or diluted to 10% vol/vol with MeOH. A small number of LC/MS method development runs were also performed using the first fraction (Jeffery 2008) of a commercial, bottled Merlot wine ("Monkey Bay" Merlot, Hawke's Bay 2012, 12.5 % alc. vol.). The 3-year old Merlot was found to be rather less suitable for anthocyanin LC/MS method development than the young Pinot Noir sample since it contained relatively low concentrations of free anthocyanins, giving poor signal to noise ratios in the resultant chromatograms (see Section 3.07).

The LC/MS runs discussed in the results section include the HPLC method (as programmed input and a graph of Solvent B % vs. time) and other relevant conditions: sample, injection volume, flow rate (total column flow, sum of A + B), column oven temperature, acid type and strength. The eluted anthocyanins were detected using the MS detector in positive SIM mode with a channel set to the parent ion of each anthocyanin. In order of elution these were Dl-3-Glu ($m/z = 465$), Cy-3-Glu ($m/z = 449$), Pt-3-Glu ($m/z = 479$), Pn-3-Glu ($m/z = 463$) and Mv-3-Glu ($m/z = 493$). Pn-3-Glu and Mv-3-Glu were usually present in the largest amounts, giving the SIM mode traces with the best signal to noise ratios. The retention times (t_R) and half-height peak widths ($w_{1/2}$) were therefore calculated from the MS SIM traces of these two proximally eluting anthocyanins, and the resultant α and R_s values used as indicators of the separating power of each LC/MS method iteration. Each of the 32 LC/MS injections performed over the course of the project were assigned a unique run number, referred to in the results as R1 - R32.

3.0 Results and Discussion - Anthocyanin method development

3.01 Initial run (R1)

The initial conditions for the method development were broadly similar to those previously used for flavanol separation (Leblanc 2015).

Sample: Pinot Noir, full strength

Injection volume: 10 μ L

Flow rate: 0.2 mL/min

Oven temperature: 25 $^{\circ}$ C

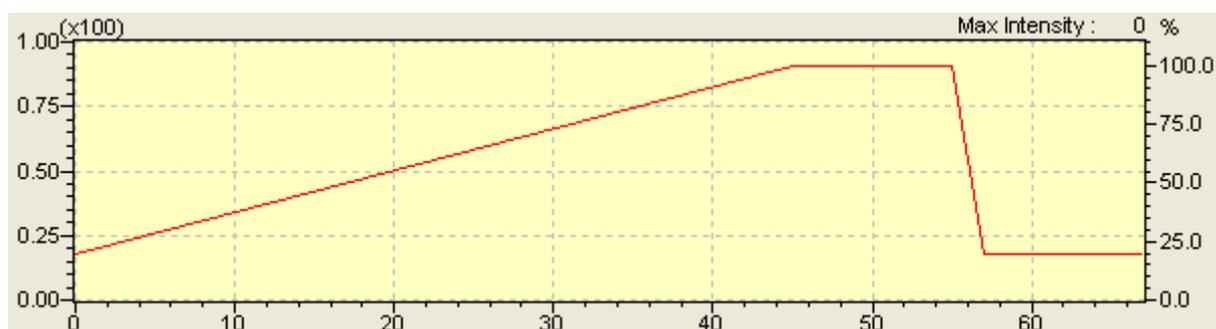
Acid: Acetic

Acid strength: 0.2% (vol/vol)

Gradient(s): 20-100% B

Method file: Alistair.lcm

Data file: asf805_1.lcd



	Time	Module	Action	Value
1	45.00	Pumps	B.Conc	100
2	55.00	Pumps	B.Conc	100
3	57.00	Pumps	B.Conc	20
4	67.00	Pumps	B.Conc	20
5	67.00	Controller	Stop	

Fig. 1: R1 solvent B gradient, LC program

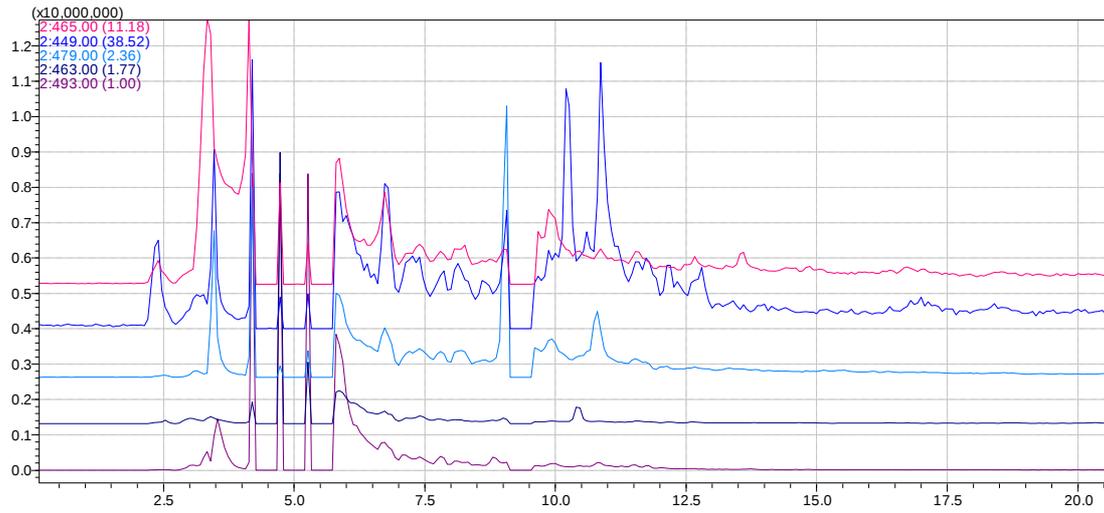


Fig. 2: R1 MS SIM anthocyanin chromatograms

α 1.00

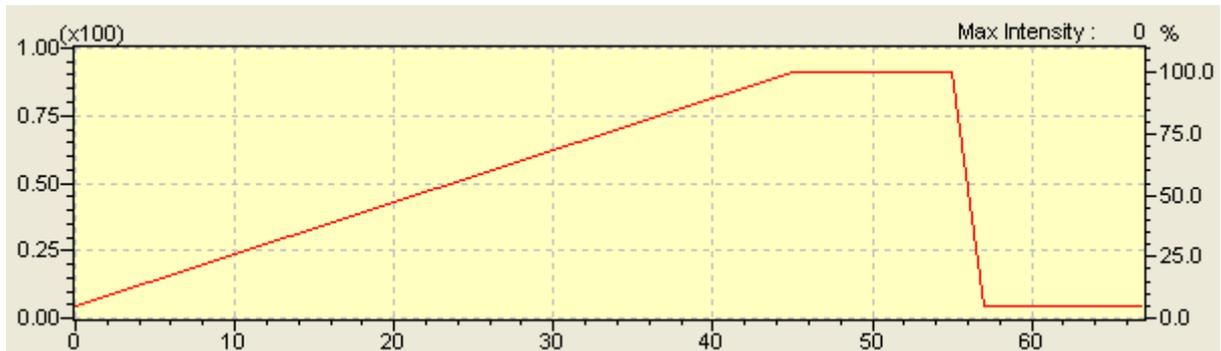
R_s n/a (MS detector cut out)

A broad gradient (20-100% B) was initially used to roughly establish how the anthocyanins behaved in the HPLC. The MS detector saturated and cut out during the peak elution, however from the very short elution time it was clear that the anthocyanins were eluting too quickly.

3.02 Lower B gradient, smaller injection volume (R2)

The very fast elution in R1 meant a gradient starting with a lower B concentration would be required. The injection volume was also reduced from 10 to 2 μL to avoid saturating the MS detector.

Sample: Pinot Noir, full strength
 Injection volume: 2 μL
 Flow rate: 0.2 mL/min
 Oven temperature: 25 $^{\circ}\text{C}$
 Acid: Acetic
 Acid strength: 0.2% (vol/vol)
 Gradient(s): 5-100% B
 Method file: asf806_1.lcm
 Data file: asf806_1.lcd



	Time	Module	Action	Value
1	45.00	Pumps	B.Conc	100
2	55.00	Pumps	B.Conc	100
3	57.00	Pumps	B.Conc	5
4	67.00	Pumps	B.Conc	5
5	67.00	Controller	Stop	

Fig. 3 : R2 solvent B gradient, LC program

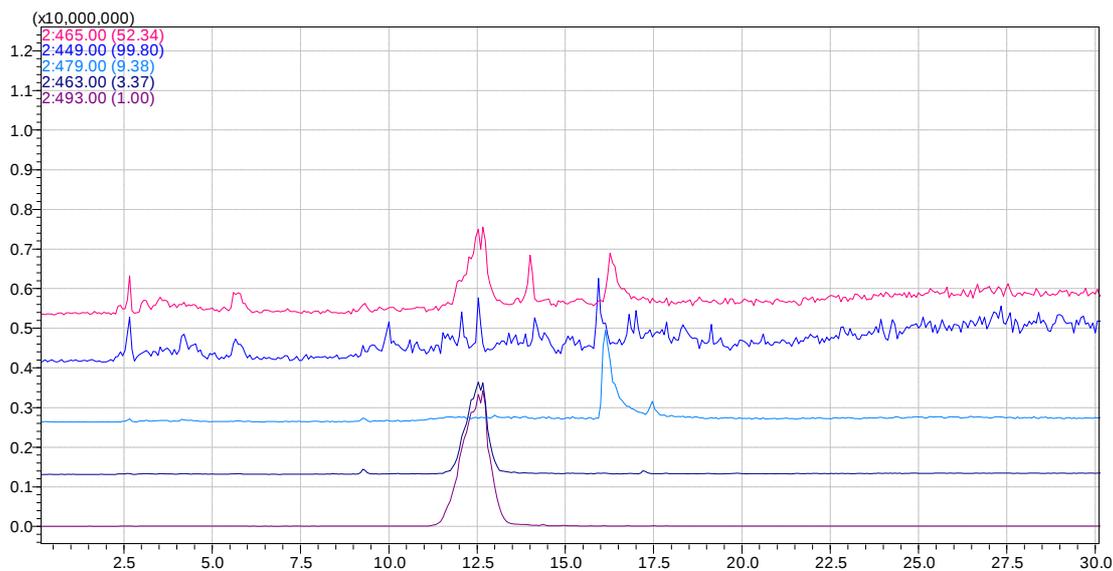


Fig. 4: R2 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) vs Mv-3-Glu ($m/z = 493$):

Separation factor (α): 0.99

Resolution (R_s): -0.10

There is effectively no separation between anthocyanins under these conditions. The $m/z = 465$ and 449 channels are shown scaled by 52 and 100 times, respectively, either due to very small amounts of Dl-3-Glu and Cy-3-Glu in the sample or due to poor detection under the conditions of this run. The Pn-3-Glu ($m/z = 463$) and Mv-3-Glu ($m/z = 493$) peaks are somewhat non-Gaussian. The tailing peak at ca. 16 min in the $m/z = 479$ channel elutes at a significantly later time than Dl-3-Glu, Pn-3-Glu or Mv-3-Glu and is unlikely to represent petunidin-3-*O*-glucoside.

3.03 Slower B gradient (R3)

The chromatogram from R2 showed anthocyanins eluted at low B concentrations, so the method used for R3 incorporated a slow B gradient from 0-15%, followed by a faster 15-40% B gradient.

Sample: Pinot Noir, full strength

Injection volume: 2 μ L

Flow rate: 0.2 mL/min

Oven temperature: 25 $^{\circ}$ C

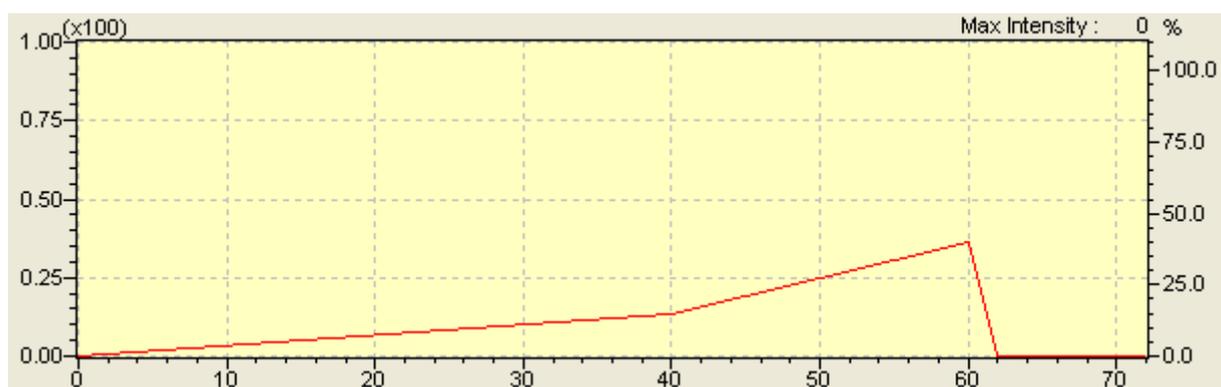
Acid: Acetic

Acid strength: 0.2% (vol/vol)

Gradient(s): 0-15-40% B

Method file: asf806_2.lcm

Data file: asf806_2.lcd



	Time	Module	Action	Value
1	40.00	Pumps	B.Conc	15
2	60.00	Pumps	B.Conc	40
3	62.00	Pumps	B.Conc	0
4	72.00	Pumps	B.Conc	0
5	72.00	Controller	Stop	

Fig. 5: R3 solvent B gradient, LC program

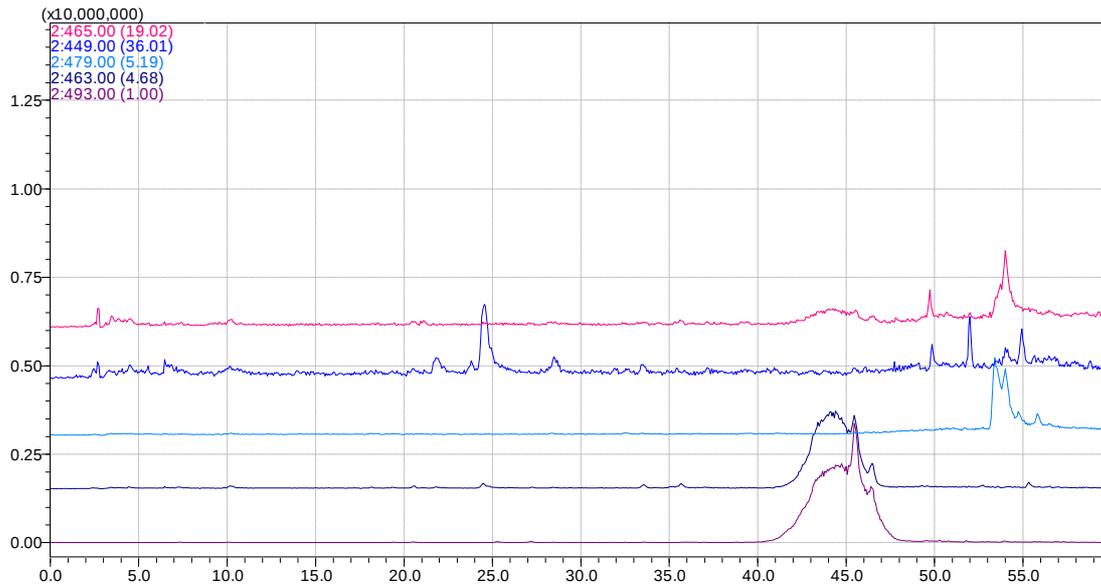


Fig. 6: R3 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) vs Mv-3-Glu ($m/z = 493$):

Separation factor (α): 1.00

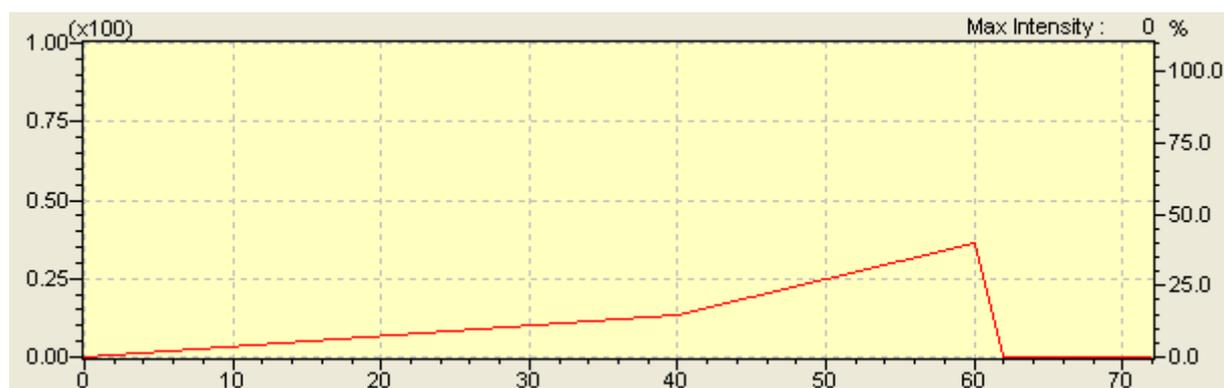
Resolution (R_s): 0

Similarly to R2, there is effectively no separation between anthocyanins despite the slower B gradient.

3.04 Higher flow rate (R4)

The next variable altered was the flow rate. The flow rate was doubled to 0.4 mL/min. All other conditions were identical to R3.

Sample: Pinot Noir, full strength
 Injection volume: 2 μ L
 Flow rate: 0.4 mL/min
 Oven temperature: 25 $^{\circ}$ C
 Acid: Acetic
 Acid strength: 0.2% (vol/vol)
 Gradient(s): 0-15-40% B
 Method file: asf807_1.lcm (identical to asf806_2.lcm)
 Data file: asf807_1.lcd



	Time	Module	Action	Value
1	40.00	Pumps	B.Conc	15
2	60.00	Pumps	B.Conc	40
3	62.00	Pumps	B.Conc	0
4	72.00	Pumps	B.Conc	0
5	72.00	Controller	Stop	

Fig. 7 : R4 solvent B gradient, LC program

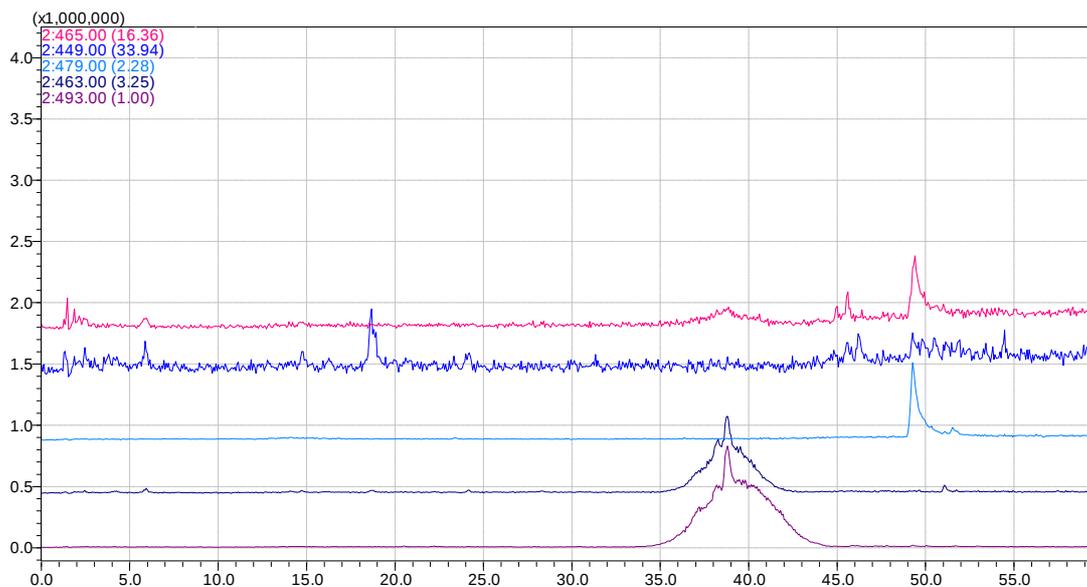


Fig. 8: R4 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) and Mv-3-Glu ($m/z = 493$):

Separation factor (α): 1.00

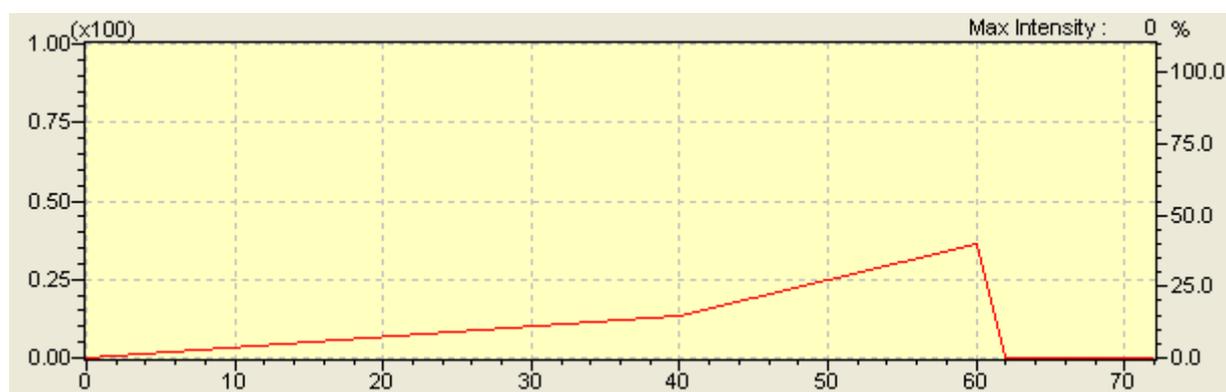
Resolution (R_s): 0

The increased 0.4 mL/min flow rate resulted in a slight decrease in t_R for the three observed anthocyanins compared with R3 (R3 t_R : 44.40 min; R4 t_R : 38.80 min), and an approximate doubling of HPLC pressure (R3 pressure at 44.40 min: 86 bar; R4 pressure at 38.80 min: 180 bar), but no separation. This was the highest pressure recorded for any of the runs in the project, and was well below the instrument maximum of 300 bar. Flow rates above 0.2 mL/min required use of a splitter after the HPLC column since the ESI inlet employed in the MS system could only evaporate 0.2 mL/min. Since the higher flow rate employed in R4 did not provide any improvement to the anthocyanin separation, a flow rate of 0.2 mL/min was used for subsequent LC/MS method development runs to avoid the time-consuming step of manually setting split ratios each time the instrument is used.

3.05 Higher column oven temperature (R5)

The next variable to be altered was the column oven temperature. Increasing the temperature of the HPLC column results in lower solvent viscosity and a lower column pressure for a given flow rate. All other conditions were identical to R3.

Sample: Pinot Noir, full strength
 Injection volume: 2 μ L
 Flow rate: 0.2 mL/min
 Oven temperature: 50 $^{\circ}$ C
 Acid: Acetic
 Acid strength: 0.2% (vol/vol)
 Gradient(s): 0-15-40% B
 Method file: asf807_2.lcm (identical to asf806_2.lcm)
 Data file: asf807_2.lcd



	Time	Module	Action	Value
1	40.00	Pumps	B.Conc	15
2	60.00	Pumps	B.Conc	40
3	62.00	Pumps	B.Conc	0
4	72.00	Pumps	B.Conc	0
5	72.00	Controller	Stop	

Fig. 9: R5 solvent B gradient, LC program

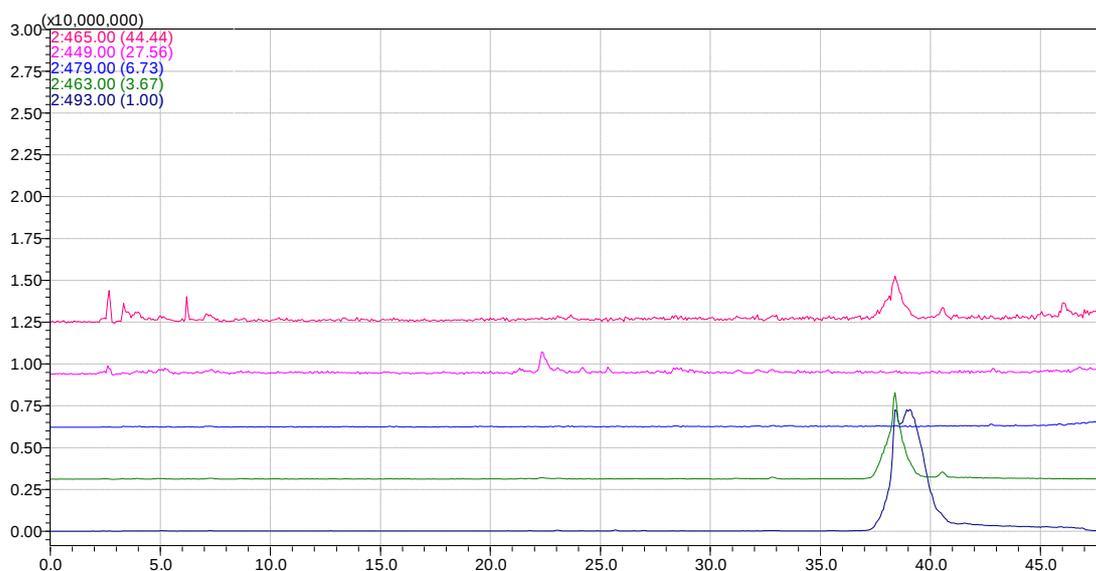


Fig. 10: R5 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) vs Mv-3-Glu ($m/z = 493$):

Separation factor (α): 1.02

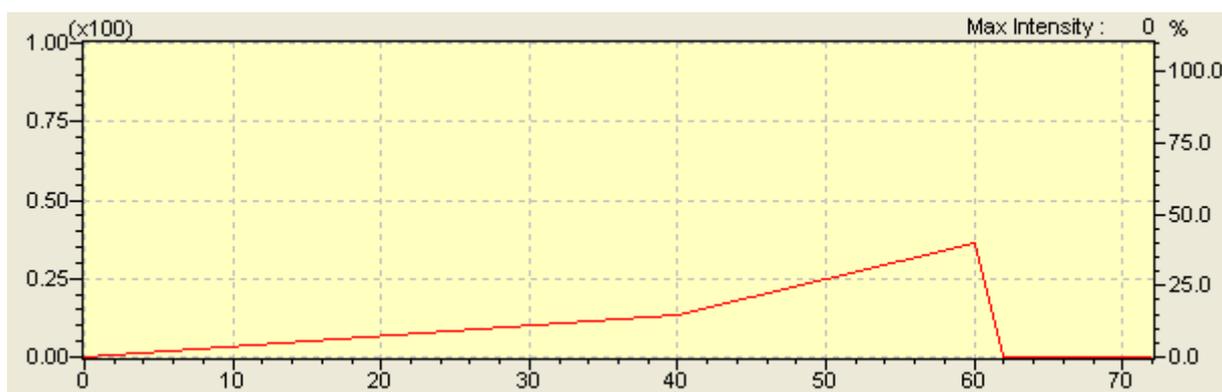
Resolution (R_s): n/a

The higher oven temperature resulted in a small separation between Pn-3-Glu and Mv-3-Glu. However the non-Gaussian peak shape of Pn-3-Glu precluded a meaningful half-height peak width from being measured, so R_s could not be determined. In light of the small but positive effect on separation, subsequent method development runs were conducted using an oven temperature of 50 °C.

3.06 Stronger acetic acid (R6)

The next iteration used an increased strength of acetic acid. All other conditions were identical to R5.

Sample: Pinot Noir, full strength
 Injection volume: 2 μ L
 Flow rate: 0.2 mL/min
 Oven temperature: 50 $^{\circ}$ C
 Acid: Acetic
 Acid strength: 1% (vol/vol)
 Gradient(s): 0-15-40% B
 Method file: asf807_2.lcm (identical to asf806_2.lcm)
 Data file: asf810_1.lcd



	Time	Module	Action	Value
1	40.00	Pumps	B.Conc	15
2	60.00	Pumps	B.Conc	40
3	62.00	Pumps	B.Conc	0
4	72.00	Pumps	B.Conc	0
5	72.00	Controller	Stop	

Fig. 11: R6 solvent B gradient, LC program

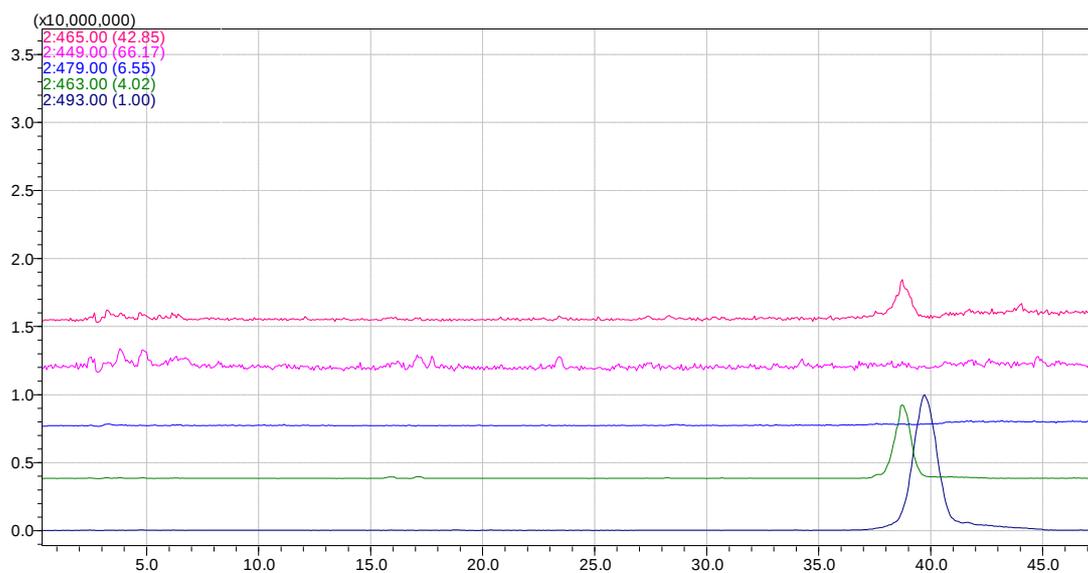


Fig. 12: R6 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) vs Mv-3-Glu ($m/z = 493$):

Separation factor (α): 1.03

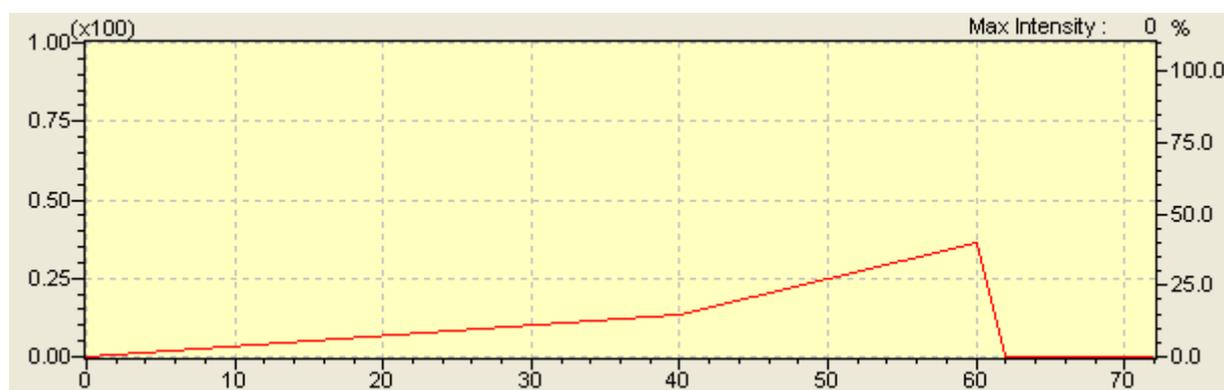
Resolution (R_s): 1.06

Increasing the level of acetic acid in solvents A and B from 0.2% to 1% resulted in an improvement in separation factor from 1.02 to 1.03. The R_s value of 1.06 was the highest amongst the methods applied to date, though less than that required to be considered baseline separated ($R_s = 1.6-2.0$).

3.07 Alternative sample - Merlot (R7)

At this point the best method developed to date was used to compare the quality of the anthocyanin chromatogram of Pinot Noir fraction #1 used up till this point with fraction #1 of a commercial Merlot ("Monkey Bay" Merlot, Hawke's Bay 2012, 12.5 % alc. vol.). The Merlot had been fractionated identically (Jeffery 2008). All other conditions were identical to R6.

Sample: Merlot, full strength
 Injection volume: 2 μ L
 Flow rate: 0.2 mL/min
 Oven temperature: 50 $^{\circ}$ C
 Acid: Acetic
 Acid strength: 1% (vol/vol)
 Gradient(s): 0-15-40% B
 Method file: asf807_2.lcm (identical to asf806_2.lcm)
 Data file: asf810_2.lcd



	Time	Module	Action	Value
1	40.00	Pumps	B.Conc	15
2	60.00	Pumps	B.Conc	40
3	62.00	Pumps	B.Conc	0
4	72.00	Pumps	B.Conc	0
5	72.00	Controller	Stop	

Fig. 13: R7 solvent B gradient, LC program

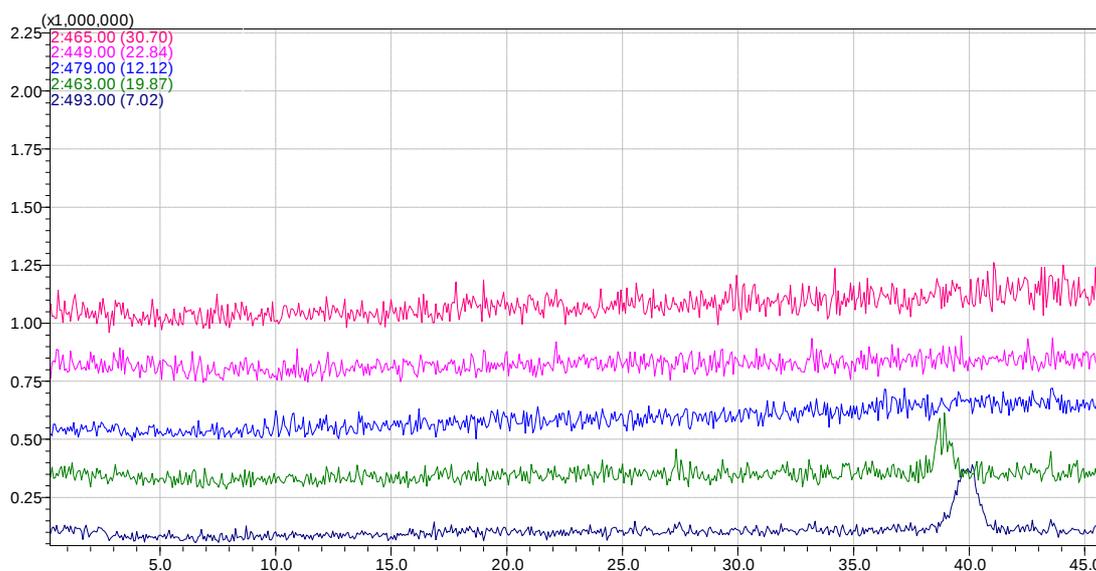


Fig. 14: R7 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) vs Mv-3-Glu ($m/z = 493$):

Separation factor (α): 1.03

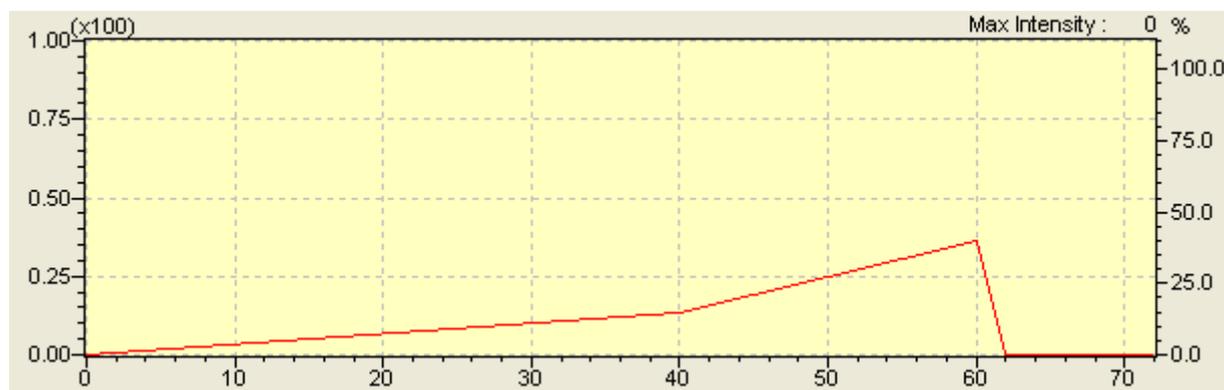
Resolution (R_s): n/a

A significant reduction in signal-to-noise is evident compared with the MS SIM chromatogram of the Pinot Noir sample (R6). No DI-3-Glu is observed in the Merlot sample and the two strongest peaks, Pn-3-Glu and Mv-3-Glu, are magnified by respective factors of 19.87 and 7.02 in this run, compared to 4.02 and 1.00 in R6. This indicates that the relatively old 2012 Merlot sample contains significantly lower amounts of anthocyanins than the Pinot Noir sample which was sampled very shortly after vinification in 2014. This result is consistent with the reduction of free anthocyanin-3-*O*-glycoside concentrations in aged wine, resulting from the formation of polymeric pigments, pyranoanthocyanins and other products (Scollary 2010). For the purposes of the current project this result confirmed the Pinot Noir sample as the preferred source of free anthocyanins, and it was used either neat or diluted for the remainder of anthocyanin method development runs.

3.08 Reducing sample injection volume (R8)

Although there was no specific indication of column overloading (e.g. peak fronting, ~Dong) in the anthocyanin SIM peaks observed this far, we wished to rule it out as a possible contributor to peak broadening before continuing with the method development. R8 was performed using an injection volume of 0.4 μL , and all other conditions were identical to R6.

Sample: Pinot Noir, full strength
 Injection volume: 0.4 μL
 Flow rate: 0.2 mL/min
 Oven temperature: 50 $^{\circ}\text{C}$
 Acid: Acetic
 Acid strength: 1% (vol/vol)
 Gradient(s): 0-15-40% B
 Method file: asf807_2.lcm (identical to asf806_2.lcm)
 Data file: asf810_3.lcd



	Time	Module	Action	Value
1	40.00	Pumps	B.Conc	15
2	60.00	Pumps	B.Conc	40
3	62.00	Pumps	B.Conc	0
4	72.00	Pumps	B.Conc	0
5	72.00	Controller	Stop	

Fig. 15: R8 solvent B gradient, LC program

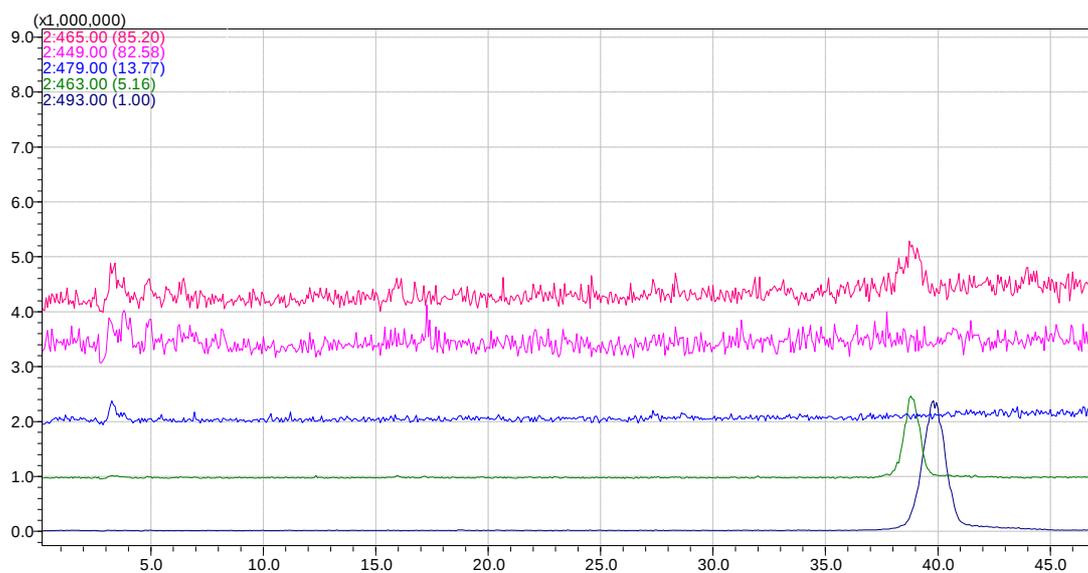


Fig. 16: R8 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) vs Mv-3-Glu ($m/z = 493$):

Separation factor (α): 1.03

Resolution (R_s): 1.03

The reduced, 0.4 μL sample injection used in 3.08 did not significantly reduce the half-height peak widths for either Pn-3-Glu or Mv-3-Glu compared with the 2 μL injection used in R6 (Pn-3-Glu half-height peak width: 0.75 min (R6), 0.83 min (R8); Mv-3-Glu half-height peak width: 1.14 min (R6), 1.12 min (R8)).

3.09 Solvent B gradient variations - isocratic/gradient elution (R9)

The B solvent gradient was altered to investigate whether an isocratic solvent mixture at a low B solvent strength might promote separation. All other conditions were identical to R6.

Sample: Pinot Noir, full strength

Injection volume: 2 μ L

Flow rate: 0.2 mL/min

Oven temperature: 50 $^{\circ}$ C

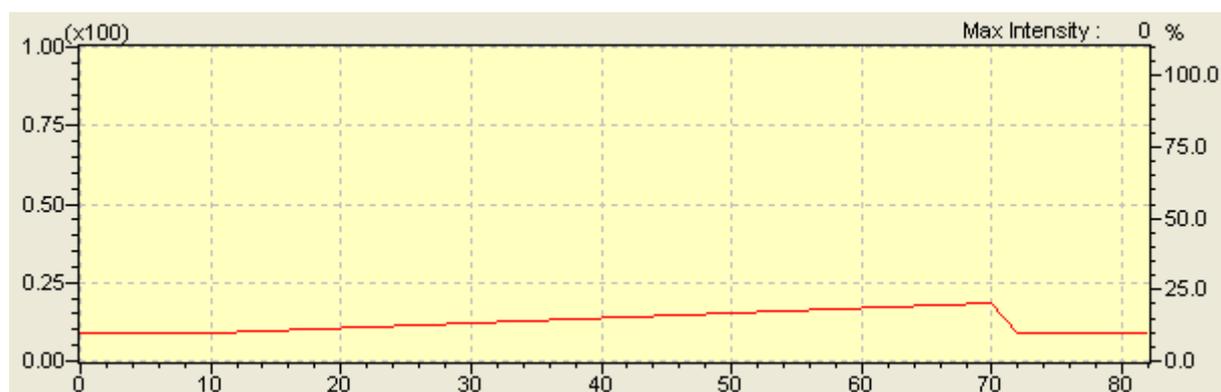
Acid: Acetic

Acid strength: 1% (vol/vol)

Gradient(s): 10(isocratic),10-20% B

Method file: asf810_1.lcm

Data file: asf811_1.lcd



	Time	Module	Action	Value
1	10.00	Pumps	B.Conc	10
2	70.00	Pumps	B.Conc	20
3	72.00	Pumps	B.Conc	10
4	82.00	Pumps	B.Conc	10
5	82.00	Controller	Stop	

Fig. 17: R9 solvent B gradient, LC program

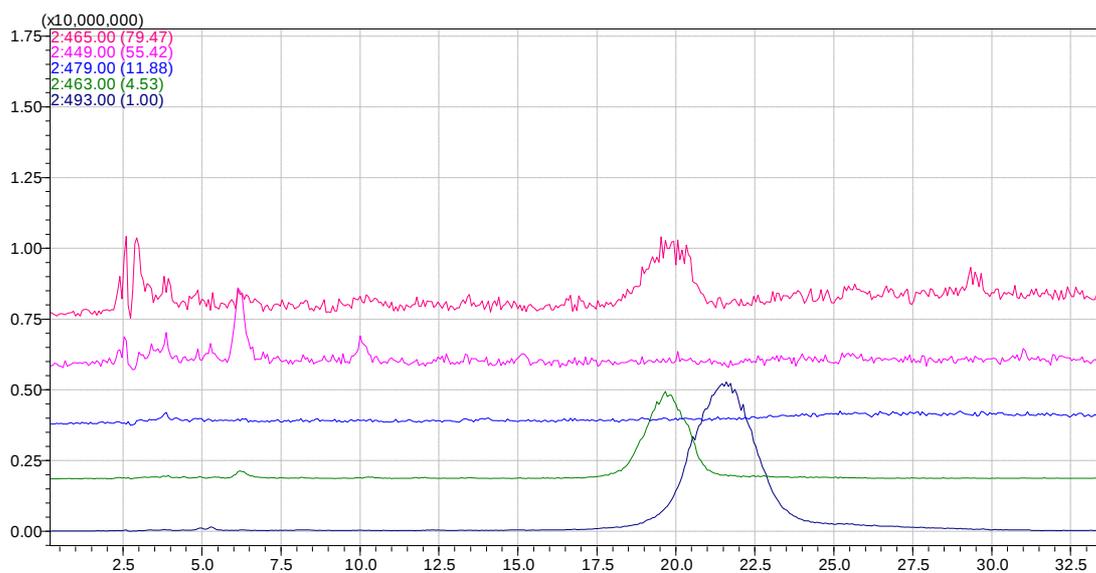


Fig. 18: R9 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) vs Mv-3-Glu ($m/z = 493$):

Separation factor (α): 1.11

Resolution (R_s): 0.96

The isocratic/slow gradient produced lower retention times and an improvement in α (1.11) compared with R6 (1.03). However, the concomitant increase in peak width resulted in a lower resolution (R6 R_s : 1.06; R9 R_s : 0.96).

3.10 Solvent B gradient variations - purely isocratic elution (R10)

Since the anthocyanins are eluting very closely an isocratic elution was performed at 11% B, which is the approximate B concentration at which anthocyanins eluted in R9. All other conditions were identical to R6.

The method used here incorporates an initial, short (2 minute) period at 0% B before changing to the target 11% B. This means that the sample will become immobilised at the head of the column upon injection, thus eliminating the possibility of pre-column broadening, and is explored further in R18.

Sample: Pinot Noir, full strength

Injection volume: 2 μ L

Flow rate: 0.2 mL/min

Oven temperature: 50 $^{\circ}$ C

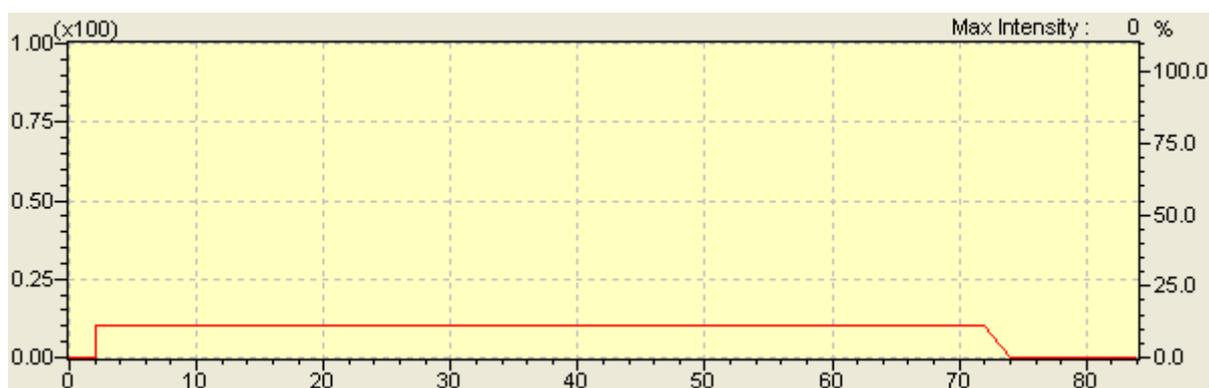
Acid: Acetic

Acid strength: 1% (vol/vol)

Gradient(s): 11% B (isocratic)

Method file: asf811_1.lcm

Data file: asf811_2.lcd



	Time	Module	Action	Value
1	2.00	Pumps	B.Conc	0
2	2.01	Pumps	B.Conc	11
3	72.00	Pumps	B.Conc	11
4	74.00	Pumps	B.Conc	0
5	84.00	Controller	Stop	

Fig. 19: R10 solvent B gradient, LC program

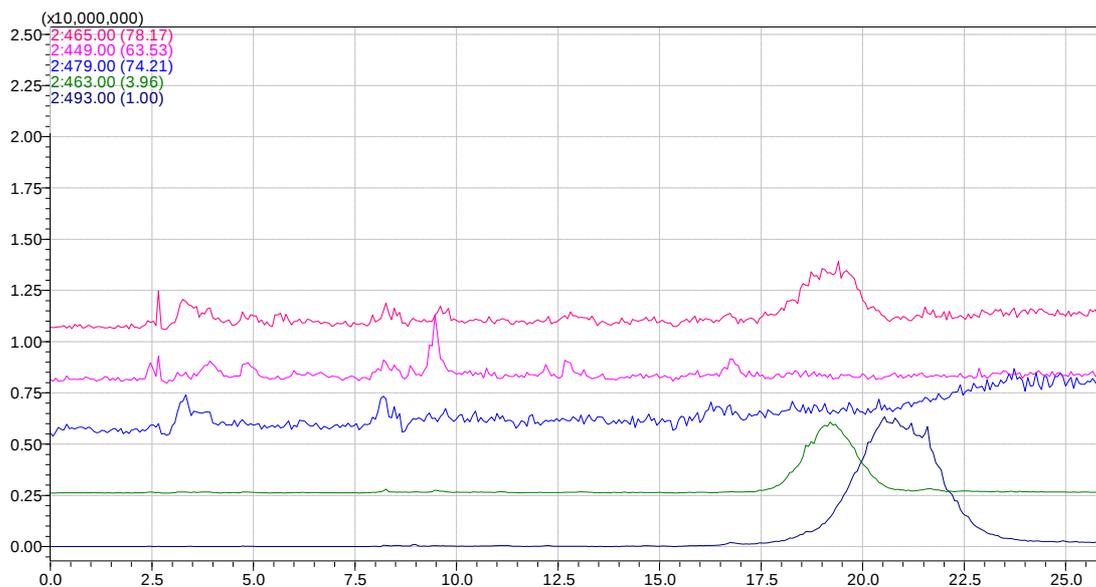


Fig. 20: R10 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) vs Mv-3-Glu ($m/z = 493$):

Separation factor (α): 1.10

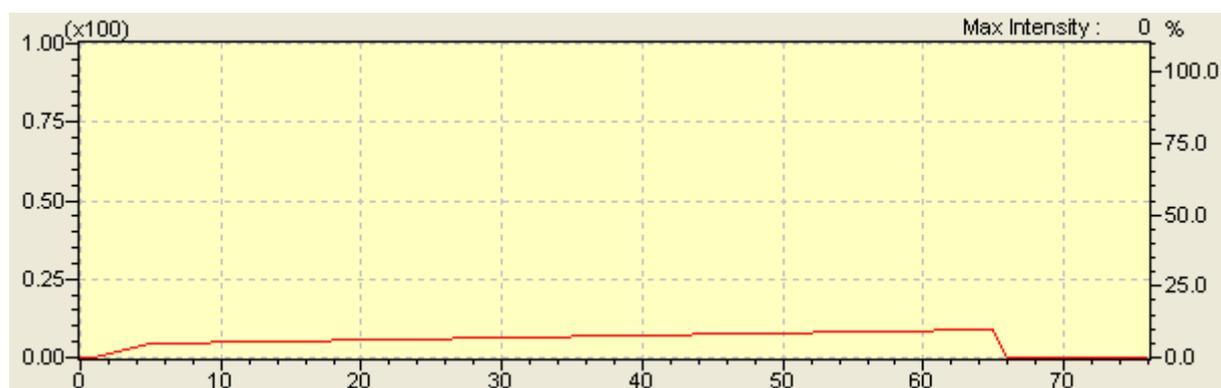
Resolution (R_s): 0.85

The 11% B isocratic elution resulted in much shorter elution times (Pn-3-Glu: 19.20 min; Mv-3-Glu: 20.86 min) than the 0-15-40% B gradient used in R6 (Pn-3-Glu: 38.73 min; Mv-3-Glu: 39.73 min). However, the peak widths (Pn-3-Glu: 1.75 min; Mv-3-Glu: 2.15 min) were considerably wider than those observed in R6 (Pn-3-Glu: 0.75 min; Mv-3-Glu: 1.14 min), resulting in lower resolution (R6 R_s : 1.06; R10 R_s : 0.85). This was thought to be due to the increasing solvent power (i.e. increasing B gradient) in R6 resulting in shorter time spent on the column and thus less opportunity for the flavylum - hemiketal equilibrium to broaden peaks. This suggests that the longer retention times noted in gradient R6 were not due to long times spent eluting through the column, but rather due to anthocyanins being held up at the head of the column during the early, more polar low-B phase of the 0-15% B gradient.

3.11 Diluted sample (R11)

During a pilot using the full strength Pinot Noir sample, the MS detector saturated causing loss of peak width and retention time information (data not shown). To avoid data loss of this nature the amounts of anthocyanins injected into the system were reduced. Injection volumes lower than 2 μL were not considered repeatable, so rather than using small injection volumes (like the 0.4 μL used in R8), the full strength Pinot Noir sample was diluted 10:1 with methanol, with a 2 μL injection volume. R11 was performed to subjectively check the chromatogram signal-to-noise using this diluted sample using the same method employed in R9. All other conditions were identical to R9.

Sample: Pinot Noir, 10:1 dilution in methanol
 Injection volume: 2 μL
 Flow rate: 0.2 mL/min
 Oven temperature: 50 °C
 Acid: Acetic
 Acid strength: 1% (vol/vol)
 Gradient(s): 10(isocratic),10-20% B
 Method file: asf810_1.lcm
 Data file: asf812_1.lcd



	Time	Module	Action	Value
1	1.00	Pumps	B.Conc	0
2	5.00	Pumps	B.Conc	5
3	65.00	Pumps	B.Conc	10
4	66.00	Pumps	B.Conc	0
5	76.00	Controller	Stop	

Fig. 21: R11 solvent B gradient, LC program

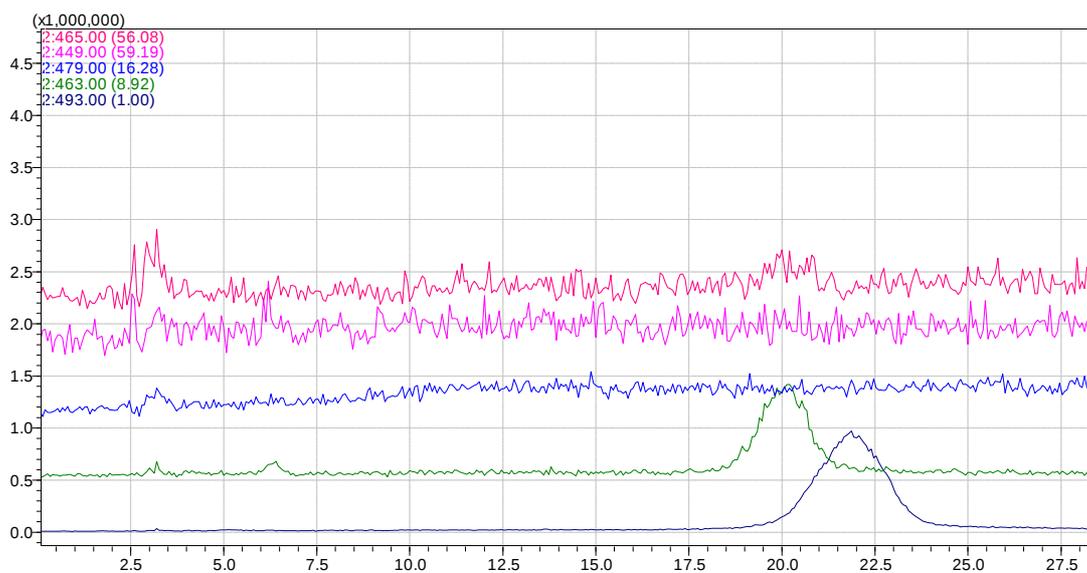


Fig. 22: R11 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) vs Mv-3-Glu ($m/z = 493$):

Separation factor (α): 1.11

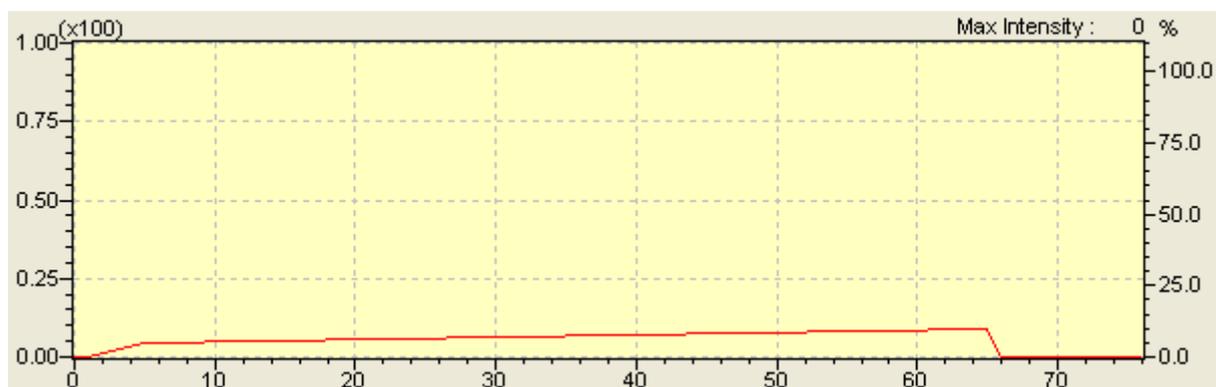
Resolution (R_s): 0.99

The separation factor (1.11) and resolution (0.99) are essentially identical to those obtained using the full strength sample in R9 (1.11 and 0.96, respectively). The poorer signal-to-noise ratio in R11 resulted in loss of the D1-3-Glu chromatogram, however the Pn-3-Glu and Mv-3-Glu peaks being used to assess the method development iterations are still clear. Quantitation of the minor anthocyanins would be revisited once a successful separation method had been developed using the diluted sample.

3.12 Slow solvent B gradient (R12)

A 5-10% B gradient was employed as a compromise between R6 (0-15-40% B) and R10 (isocratic 11% B), targeting the B concentration where the anthocyanins begin elution. The gradient slope was chosen to be very slow (0.083% B/min).

Sample: Pinot Noir, 10:1 dilution in methanol
 Injection volume: 2 μ L
 Flow rate: 0.2 mL/min
 Oven temperature: 50 $^{\circ}$ C
 Acid: Acetic
 Acid strength: 1% (vol/vol)
 Gradient(s): 5-10% B (0.083% B/min)
 Method file: asf.lcm
 Data file: asf812_2.lcd



	Time	Module	Action	Value
1	1.00	Pumps	B.Conc	0
2	5.00	Pumps	B.Conc	5
3	65.00	Pumps	B.Conc	10
4	66.00	Pumps	B.Conc	0
5	76.00	Controller	Stop	

Fig. 23: R12 solvent B gradient, LC program

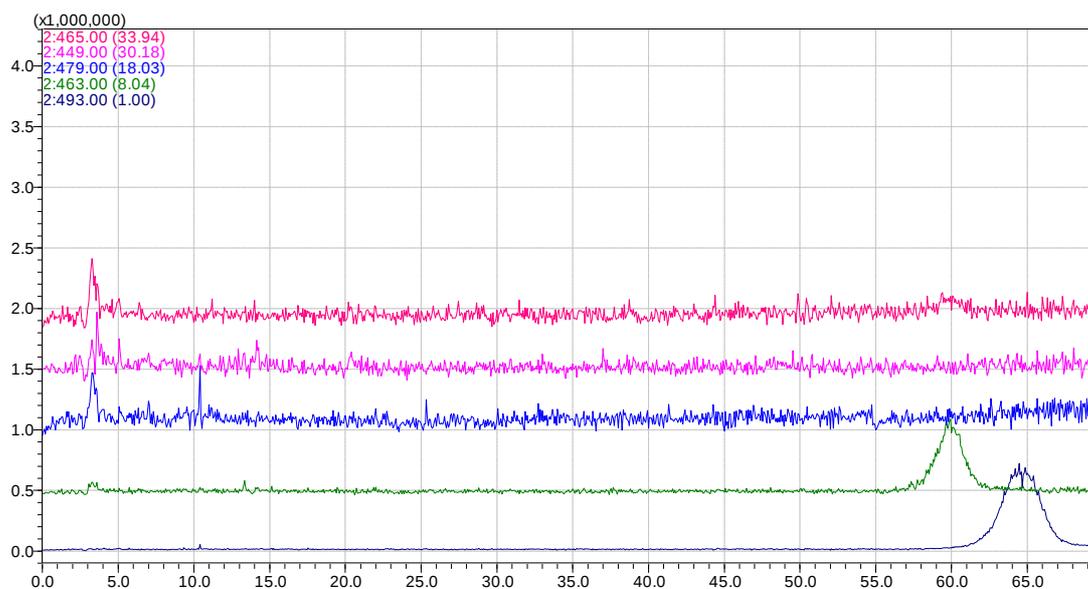


Fig. 24: R12 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) vs Mv-3-Glu ($m/z = 493$):

Separation factor (α): 1.08

Resolution (R_s): 2.01

The very slow B gradient produced a successful baseline separation between Pn-3-Glu and Mv-3-Glu ($R_s = 2.01$, at the high end of the 1.6-2.0 range normally considered baseline separated). However this separation required over an hour of elution. Also, looking at the noisy $m/z = 465$ trace, it seems Dl-3-Glu co-elutes with Pn-3-Glu under these conditions. This applicability of this method to the other anthocyanins would need to be verified using a stronger sample before being used as the basis for PDA detection of anthocyanins.

3.13 Slow solvent B gradient (R13)

A narrower solvent range was employed in an attempt to reduce the very long elution time observed in R12. All other conditions were identical to R12.

Sample: Pinot Noir, 10:1 dilution in methanol

Injection volume: 2 μ L

Flow rate: 0.2 mL/min

Oven temperature: 50 $^{\circ}$ C

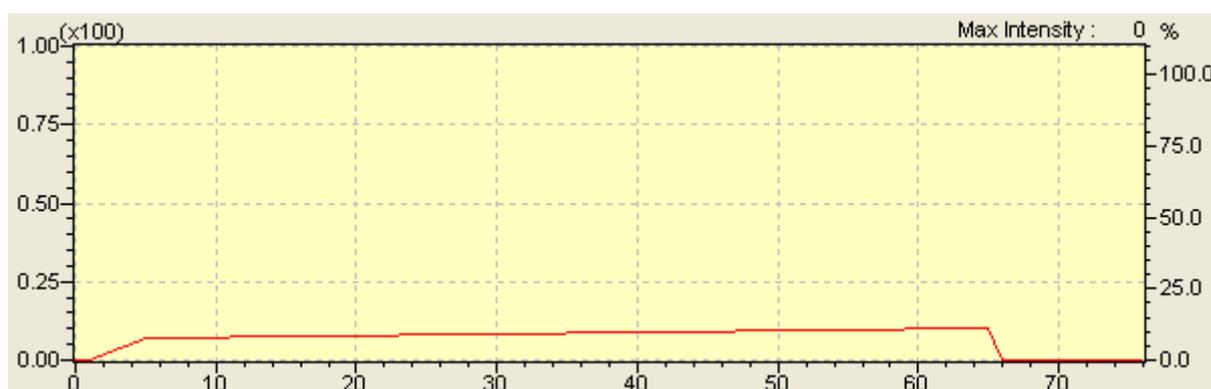
Acid: Acetic

Acid strength: 1% (vol/vol)

Gradient(s): 8-11% B (0.05% B/min)

Method file: asf812_3.lcm

Data file: asf812_3.lcd



	Time	Module	Action	Value
1	1.00	Pumps	B.Conc	0
2	5.00	Pumps	B.Conc	8
3	65.00	Pumps	B.Conc	11
4	66.00	Pumps	B.Conc	0
5	76.00	Pumps	B.Conc	0
6	76.00	Controller	Stop	

Fig. 25: R13 solvent B gradient, LC program

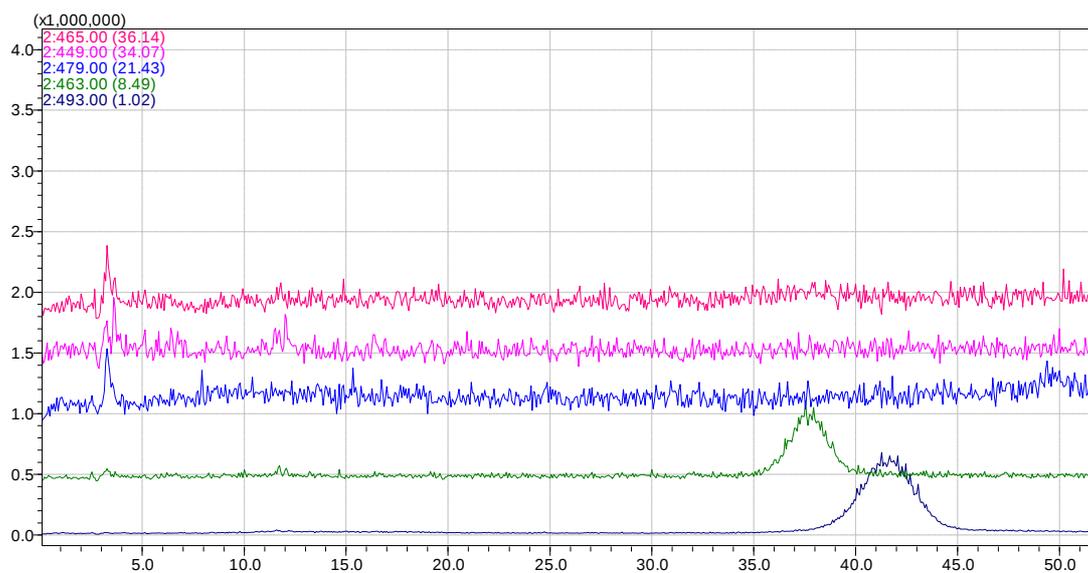


Fig. 26: R13 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) vs Mv-3-Glu ($m/z = 493$):

Separation factor (α): 1.11

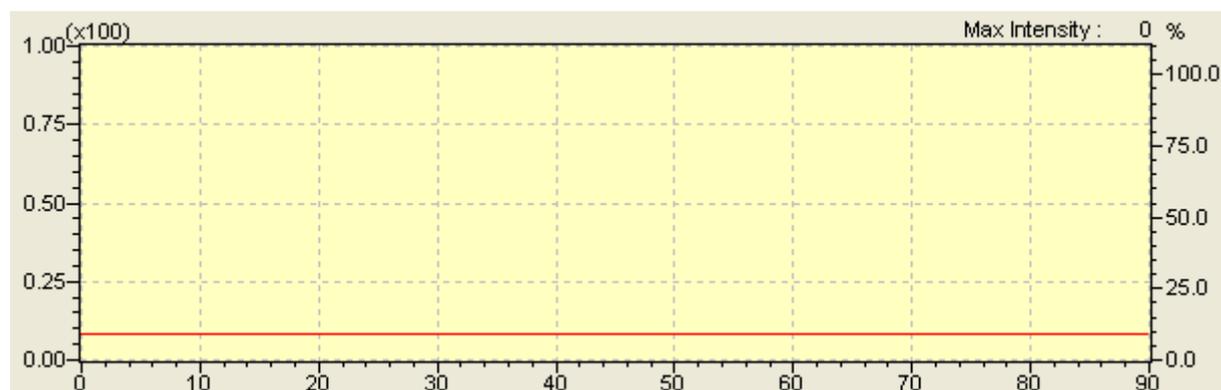
Resolution (R_s): 1.58

The retention times are lower in R13, producing a slight increase in α (R12 α : 1.08; R13 α : 1.11) but lower resolution (R12 R_s : 2.01; R13 R_s : 1.58), which might make this method marginal for quantification of individual anthocyanins by PDA.

3.14 Solvent B isocratic runs (R14, R15, R16)

A set of isocratic elutions were performed to see if the B concentration could be tailored to match the separation obtained from the very slow gradient elutions used in R12 and R13. Three isocratic elutions were performed at 9.3, 9.1 and 8.9% B. All other conditions were identical to R6.

Sample: Pinot Noir, 10:1 dilution in methanol
 Injection volume: 2 μ L
 Flow rate: 0.2 mL/min
 Oven temperature: 50 $^{\circ}$ C
 Acid: Acetic
 Acid strength: 1% (vol/vol)
 Gradient(s): 9.3% (R14), 9.1% (R15), 8.9% (R16) B, isocratic
 Method files: asf817_1.lcm (R14), asf817_2.lcm (R15), asf817_3.lcm (R16)
 Data files: asf817_1.lcd (R14), 817_2.lcd (R15), 818_1.lcd (R16)



	Time	Module	Action	Value
1	90.00	Pumps	B.Conc	9.3
2	90.00	Controller	Stop	

	Time	Module	Action	Value
1	90.00	Pumps	B.Conc	9.1
2	90.00	Controller	Stop	

	Time	Module	Action	Value
1	60.00	Pumps	B.Conc	8.9
2	60.00	Controller	Stop	

Fig. 27: R14 solvent B gradient; R14, R15, R16 LC programs

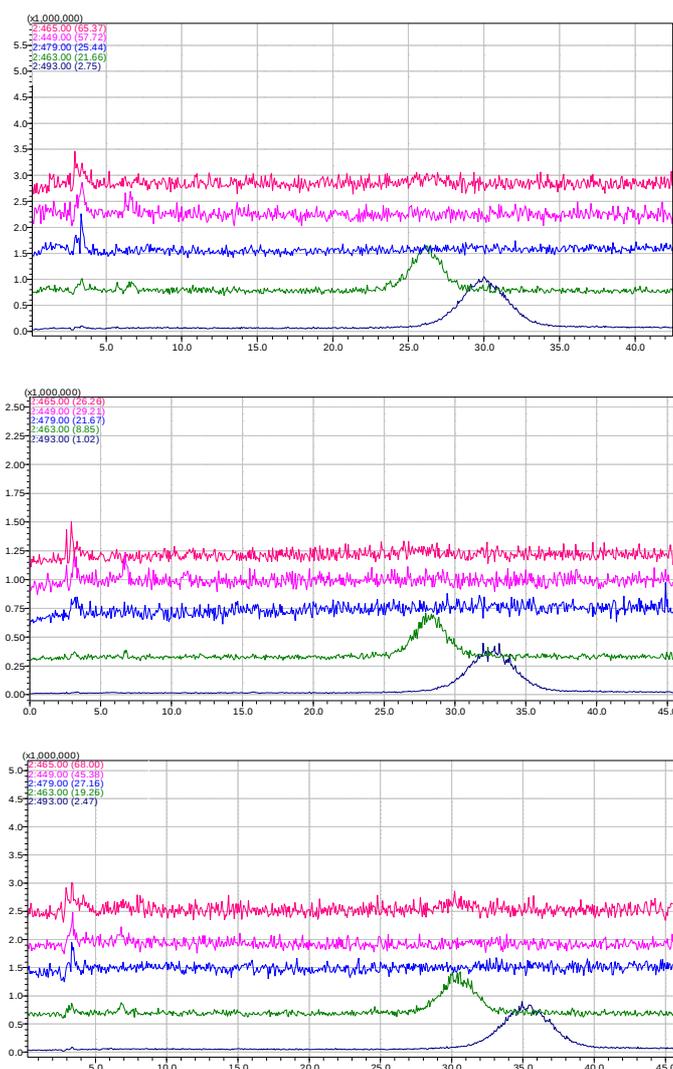


Fig. 28: R14, R15, R16 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) vs Mv-3-Glu ($m/z = 493$); 9.3, 9.1 and 8.9% B respectively:

Separation factor (α): 1.16, 1.18, 1.18

Resolution (R_s): 1.32, 1.53, 1.48

The group of isocratic elutions produced α values somewhat better than those obtained by slow gradient elution (R12 α : 1.08; R13 α : 1.11) but similar or poorer R_s values (R12 R_s : 2.01; R13 R_s : 1.58). Although the isocratic elution times were faster (desirable for minimising analysis time), gradient methods were pursued since they produced the best resolution values so far.

3.15 Stronger acetic acid (R17)

The acetic acid concentration was raised to 2% in an attempt to lower pH and reduce the line broadening influence of the hemiketal forms on anthocyanin peak widths. All other conditions were identical to R13, including the use of a slow 8-11% B gradient.

Sample: Pinot Noir, 10:1 dilution in methanol

Injection volume: 2 μ L

Flow rate: 0.2 mL/min

Oven temperature: 50 $^{\circ}$ C

Acid: Acetic

Acid strength: 2% (vol/vol)

Gradient(s): 8-11% B

Method file: asf812_3.lcm

Data file: asf818_2.lcd



	Time	Module	Action	Value
1	1.00	Pumps	B.Conc	0
2	5.00	Pumps	B.Conc	8
3	65.00	Pumps	B.Conc	11
4	66.00	Pumps	B.Conc	0
5	76.00	Pumps	B.Conc	0
6	76.00	Controller	Stop	

Fig. 29: R17 solvent B gradient, LC program

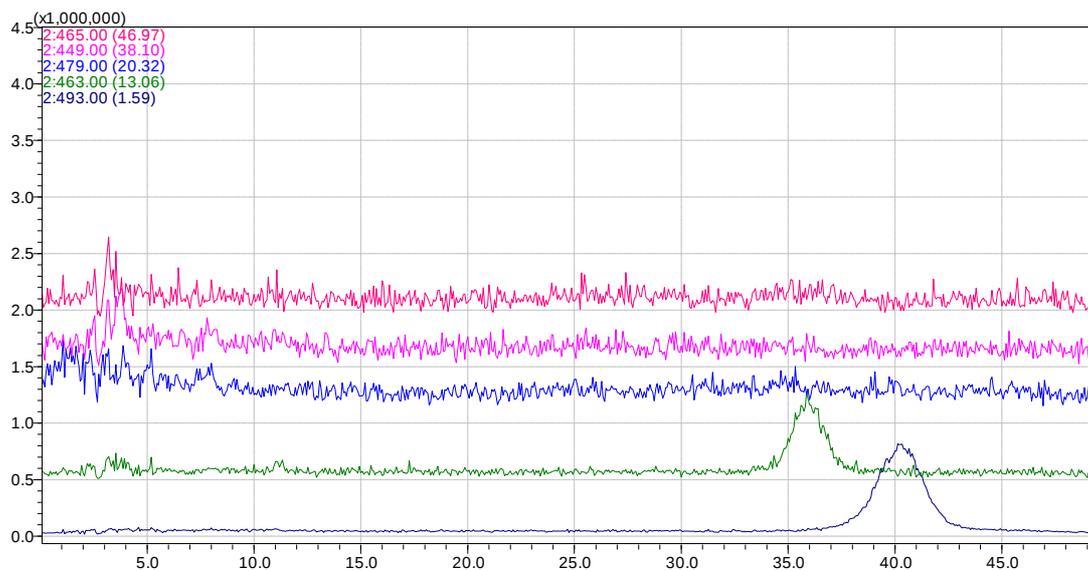


Fig. 30: R17 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) vs Mv-3-Glu ($m/z = 493$):

Separation factor (α): 1.13

Resolution (R_s): 2.18

The increase in acid concentration from 1% (R13) to 2% resulted in an increase in α from 1.11 to 1.13. Reductions in peak width for both Pn-3-Glu (R13: 1.95 min, R17: 1.56 min) and Mv-3-Glu (R13: 2.85 min, R17: 2.35 min) resulted in an increase in R_s from 1.58 to 2.18, indicating baseline separation.

3.16 Pre-column broadening (R18)

Pre-column broadening is an increase in peak width brought caused by diffusion of the sample prior to loading onto the column. The phenomenon is a result of deficiencies in instrument design, and as such it might not be expected to affect modern equipment (Dong, 2006). To see if pre-column broadening was relevant to this study, R17 was repeated using a method which started at the initial B gradient concentration rather than 0. All other conditions were identical to R17. Column conditioning was carried out at 8% B before the run, and the final B concentration (9.1%) was chosen to prepare the column for the following method.

Sample: Pinot Noir, 10:1 dilution in methanol

Injection volume: 2 μ L

Flow rate: 0.2 mL/min

Oven temperature: 50 $^{\circ}$ C

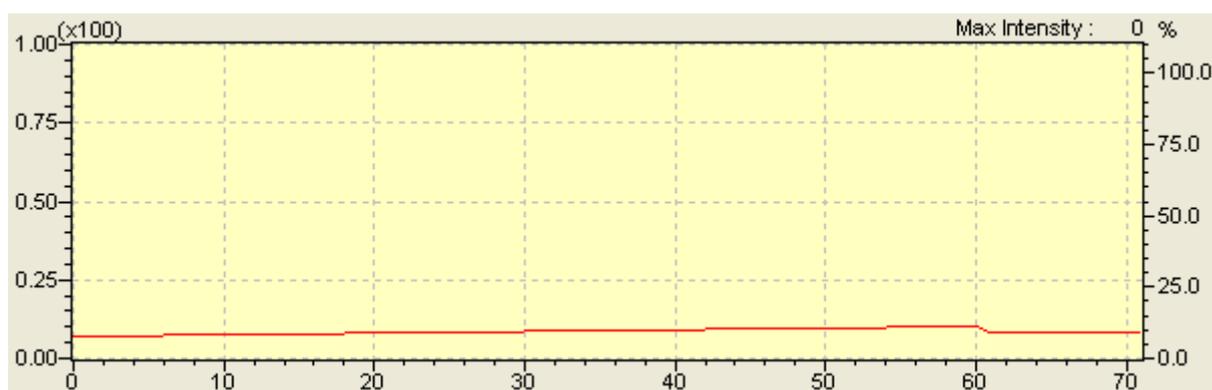
Acid: Acetic

Acid strength: 1% (vol/vol)

Gradient(s): 8-11% B

Method file: asf818_3.lcm

Data file: asf818_3.lcd



	Time	Module	Action	Value
1	60.00	Pumps	B.Conc	11
2	61.00	Pumps	B.Conc	9.1
3	71.00	Controller	Stop	

Fig. 31: R18 solvent B gradient, LC program

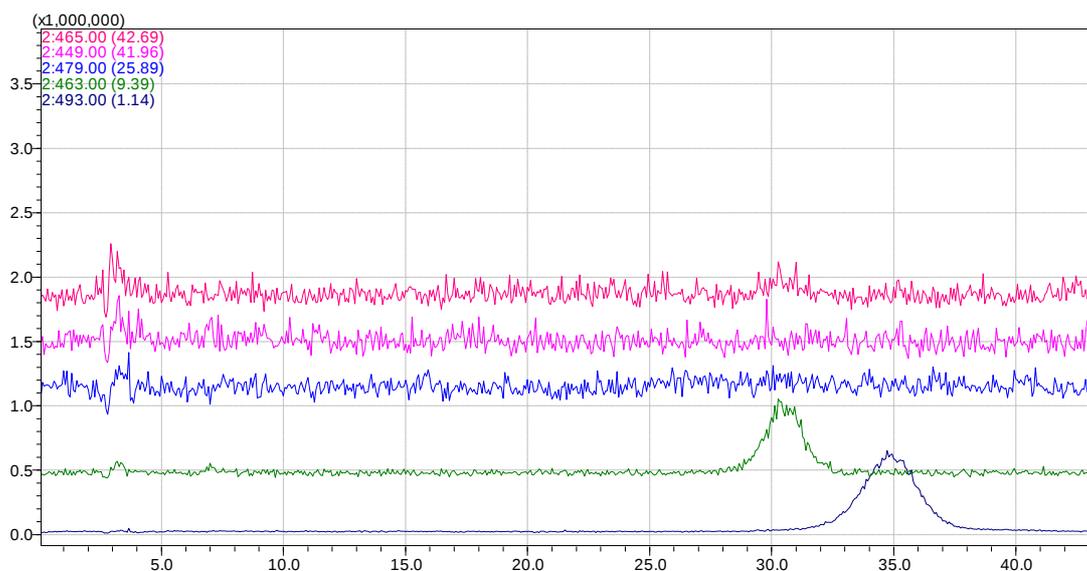


Fig. 32: R18 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) vs Mv-3-Glu ($m/z = 493$):

Separation factor (α): 1.16

Resolution (R_s): 2.13

Compared with R17, which started at 0% B, there is a slight increase in peak width for both Pn-3-Glu (R17: 1.56 min; R18: 1.72 min) and Mv-3-Glu (R17: 2.35 min; R18: 2.45 min). The extra time spent during analyte immobilisation at 0% B has the effect of reducing the separation factor since α is calculated as the *ratio* of retention times, an effect which is reflected in the lower separation factor calculated for R17 (R17 α : 1.13; R18 α : 1.16). Resolution is a more appropriate metric for comparison since R_s is calculated from the *difference* in retention times, revealing a slight advantage in starting at 0% B (R17 R_s : 2.18; R18 R_s : 2.13). In addition to a modest improvements in peak width, beginning each run with 0% B has the added procedural advantage of standardising the column conditioning between runs, eliminating the need to match the column reconditioning solvent mixture to the particular initial B concentration of the following method. This was an important factor in minimising the time required between LC/MS method development runs.

3.17 Stronger acetic acid (R19)

Acetic acid strength was increased to 10% using an 8-11% B gradient. All other conditions (except the column conditioning sequence) were identical to R17. The column conditioning introduced a period at 100% B which ensured the complete removal of all late-eluting compounds from the column, such as pyranoanthocyanins, polymeric pigments etc. This was incorporated into all subsequent methods.

Sample: Pinot Noir, 10:1 dilution in methanol

Injection volume: 2 μ L

Flow rate: 0.2 mL/min

Oven temperature: 50 $^{\circ}$ C

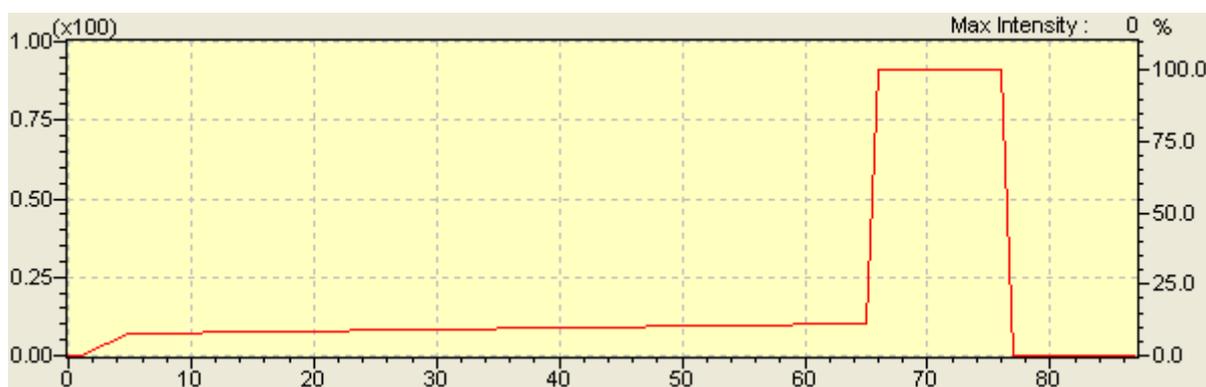
Acid: Acetic

Acid strength: 10% (vol/vol)

Gradient(s): 8-11% B

Method file: asf821_2.lcm

Data file: asf821_2.lcd



	Time	Module	Action	Value
1	1.00	Pumps	B.Conc	0
2	5.00	Pumps	B.Conc	8
3	65.00	Pumps	B.Conc	11
4	66.00	Pumps	B.Conc	100
5	76.00	Pumps	B.Conc	100
6	77.00	Pumps	B.Conc	0
7	87.00	Controller	Stop	

Fig. 33: R19 solvent B gradient, LC program

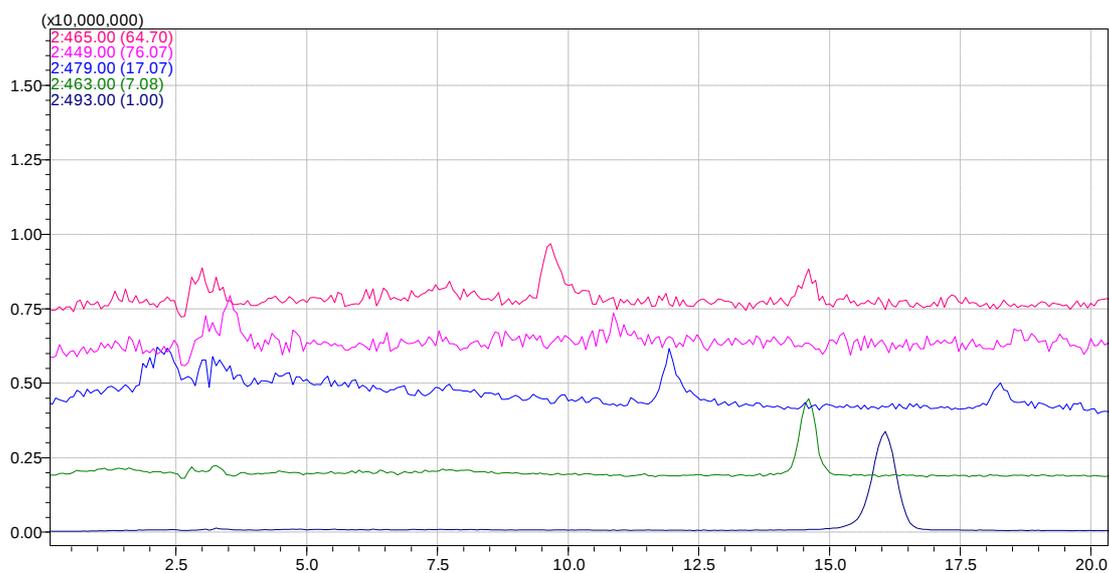


Fig. 34: R19 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) vs Mv-3-Glu ($m/z = 493$):

Separation factor (α): 1.13

Resolution (R_s): 3.36

Acetic acid at 10% improved both α and R_s over the values obtained at 2% (R17: 1.16 and 2.13, respectively). The clear signals present in the $m/z = 465$ and 479 channels are in stark contrast with the chromatogram obtained in R17, in which neither was observable. This indicates the flavylum concentrations of Dl-3-Glu and Pt-3-Glu are increased significantly at 10% acetic acid.

3.18 Acidification with formic acid (R20)

Formic acid (10%) was used to acidify solvents A and B instead of acetic acid. All other conditions were identical to R19.

Sample: Pinot Noir, 10:1 dilution in methanol

Injection volume: 2 μ L

Flow rate: 0.2 mL/min

Oven temperature: 50 $^{\circ}$ C

Acid: Formic

Acid strength: 10% (vol/vol)

Gradient(s): 8-11% B

Method file: asf821_2.lcm

Data file: asf907_1.lcd



	Time	Module	Action	Value
1	1.00	Pumps	B.Conc	0
2	5.00	Pumps	B.Conc	8
3	65.00	Pumps	B.Conc	11
4	66.00	Pumps	B.Conc	100
5	76.00	Pumps	B.Conc	100
6	77.00	Pumps	B.Conc	0
7	87.00	Controller	Stop	

Fig. 35: R20 solvent B gradient, LC program

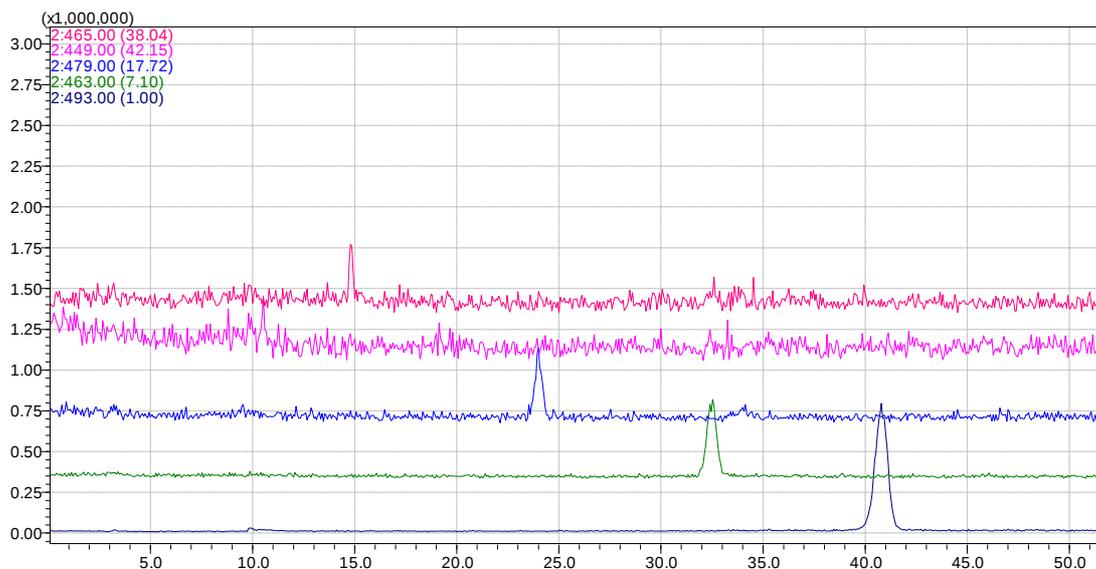


Fig. 36: R20 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) vs Mv-3-Glu ($m/z = 493$):

Separation factor (α): 1.28

Resolution (R_s): 14.40

Switching from acetic to formic acid produced a very large improvement to both α and R_s . The peak widths are similar to those observed with 10% acetic acid (e.g. R19 Mv-3-Glu $w_{1/2}$: 0.54 min; R20 Mv-3-Glu $w_{1/2}$: 0.68 min) however the five anthocyanins elute over a far wider time-frame using formic acid (R19 Mv-3-Glu t_R - Dl-3-Glu $t_R = 6.6$ min; R20 Mv-3-Glu t_R - Dl-3-Glu $t_R = 26.3$ min), resulting in much greater resolution. The individual retention times are significantly greater using 10% formic acid (e.g. R19 Mv-3-Glu $t_R = 16.08$ min; R20 Mv-3-Glu $t_R = 40.81$ min) which is likely due to the greater polarity of formic acid compared to acetic acid (dielectric constants: formic acid = $56.1 \epsilon_0$; acetic acid = $6.15 \epsilon_0$) (Lagowski, 1970). The net increase in polar character contributed to solvents A and B by substituting formic for acetic acid results in an increased affinity of the anthocyanin analytes to the C_{18} stationary phase, resulting in longer retention times.

3.19 Solvent B gradient variation (R21)

The method begins with the same 0.05%B/min slope used in R20, but stopping it at point where Mv-3-Glu eluted (9.75% B). From this point a steeper gradient was used to see if the later-eluting compounds could be separated as part of the same LC method. All other conditions were identical to R20.

Sample: Pinot Noir, 10:1 dilution in methanol

Injection volume: 2 μ L

Flow rate: 0.2 mL/min

Oven temperature: 50 °C

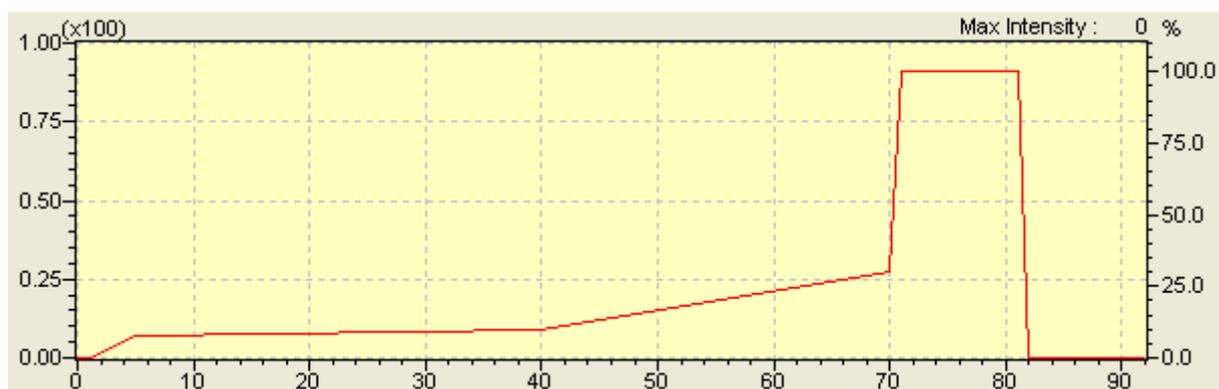
Acid: Formic

Acid strength: 10% (vol/vol)

Gradient(s): 8-9.75-30% B

Method file: asf907_2.lcm

Data file: asf907_2.lcd



	Time	Module	Action	Value
1	1.00	Pumps	B.Conc	0
2	5.00	Pumps	B.Conc	8
3	40.00	Pumps	B.Conc	9.75
4	70.00	Pumps	B.Conc	30
5	71.00	Pumps	B.Conc	100
6	81.00	Pumps	B.Conc	100
7	82.00	Pumps	B.Conc	0
8	92.00	Controller	Stop	

Fig. 37: R21 solvent B gradient, LC program

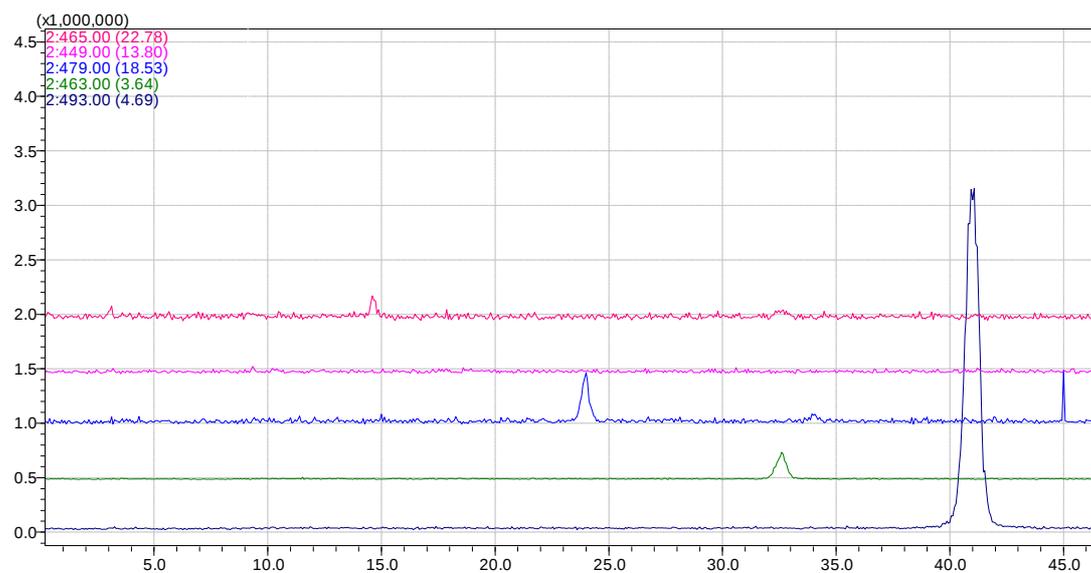


Fig. 38: R21 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) vs Mv-3-Glu ($m/z = 493$):

Separation factor (α): 1.28

Resolution (R_s): 14.74

As expected, using the same solvent B gradient as R20 produced a similar degree of anthocyanin separation. The 9.75-30% B gradient gave separation of some later-eluting compounds (see following section for late-eluting results).

3.20 Faster solvent B gradient (R22)

Having demonstrated excellent anthocyanin separation in R20 and R21, the focus of the anthocyanin method development turned to reducing the time required for analysis. R20 and R21 separated the five anthocyanins using a solvent B gradient of 0.05% B/min, producing R_s figures in excess of 14. R22 employed a faster gradient (0.10% B/min) in an effort to reduce the overall analysis time whilst still maintaining good separation. In addition, the second gradient was extended to 100% B to increase the number of late-eluting compounds separated by the method. All other conditions were identical to R20.

Sample: Pinot Noir, 10:1 dilution in methanol

Injection volume: 2 μ L

Flow rate: 0.2 mL/min

Oven temperature: 50 $^{\circ}$ C

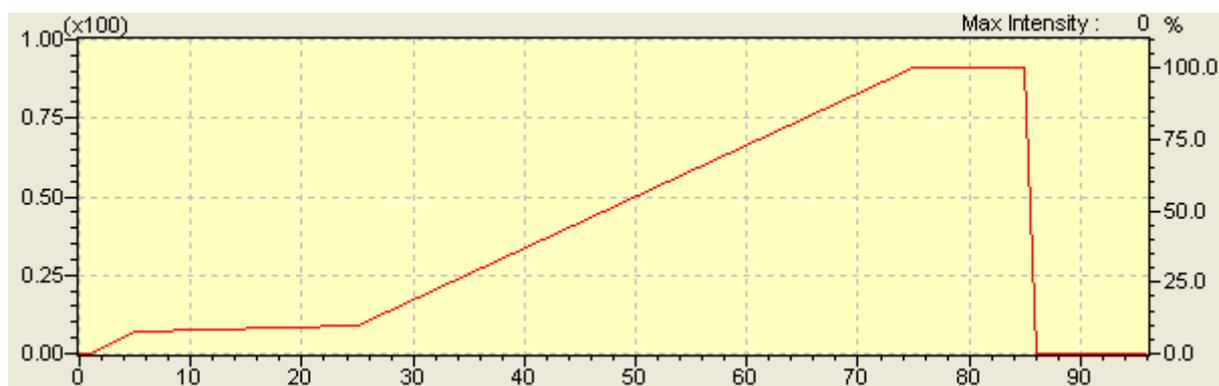
Acid: Formic

Acid strength: 10% (vol/vol)

Gradient(s): 8-10-100% B

Method file: asf907_3.lcm

Data file: asf907_3.lcd



	Time	Module	Action	Value
1	1.00	Pumps	B.Conc	0
2	5.00	Pumps	B.Conc	8
3	25.00	Pumps	B.Conc	10
4	75.00	Pumps	B.Conc	100
5	85.00	Pumps	B.Conc	100
6	86.00	Pumps	B.Conc	0
7	96.00	Controller	Stop	

Fig. 39: R22 solvent B gradient, LC program

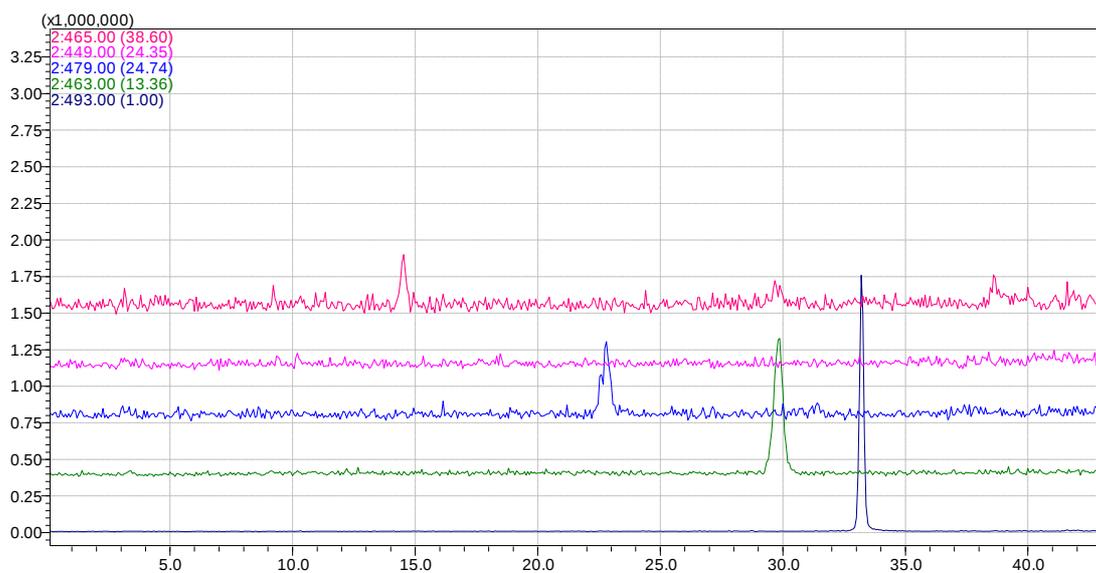


Fig. 40: R22 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) vs Mv-3-Glu ($m/z = 493$):

Separation factor (α): 1.13

Resolution (R_s): 11.72

The faster 8-10% B gradient still produced very good anthocyanin separation, requiring ca. 34 min for Mv-3-Glu to elute, compared with ca. 43 min for R20.

The second 10-100% B gradient proved successful at separating late eluting compounds (see following section for late-eluting results).

3.21 Reducing formic acid strength (R23, R24)

Having established the effectiveness of 10% formic acid as an acidifying agent, the effect of lower formic acid concentrations on anthocyanin separation was investigated. Runs were performed using 1% and 0.1% formic acid with the same initial 8-9.75% B gradient (at 0.05% B/min) employed in R21. (The second gradient extends to 60% in the method used for 0.1% formic acid, but this is only relevant to late-eluting pigments.) All other conditions were identical to R21.

Sample: Pinot Noir, 10:1 dilution in methanol
 Injection volume: 2 μ L
 Flow rate: 0.2 mL/min
 Oven temperature: 50 $^{\circ}$ C
 Acid: Formic
 Acid strength: 1% (R23), 0.1% (R24) (vol/vol)
 Gradient(s): 8-9.75-30% B (R23), 8-9.75-60% (R24)
 Method file: asf907_2.lcm (R23), asf908_2.lcm (R24)
 Data file: asf908_1.lcd (R23), asf908_2.lcd (R24)

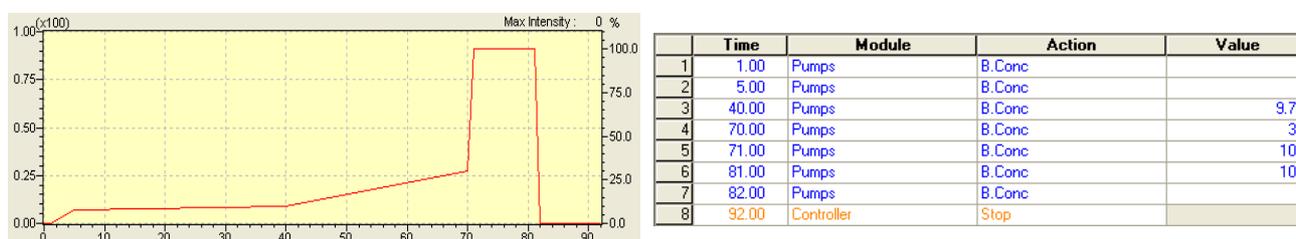


Fig. 41: R23 solvent B gradient, LC program

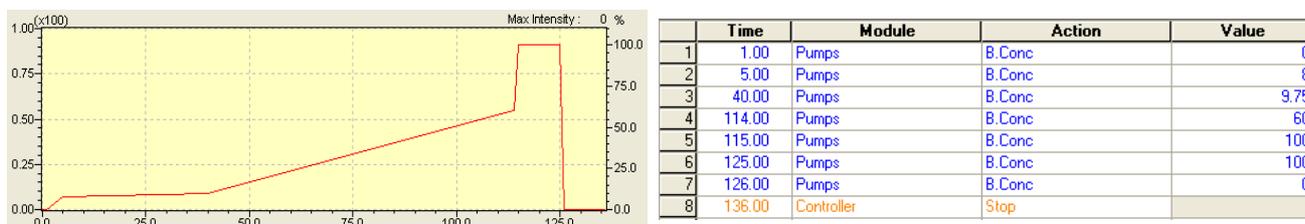


Fig. 42: R24 solvent B gradient, LC program

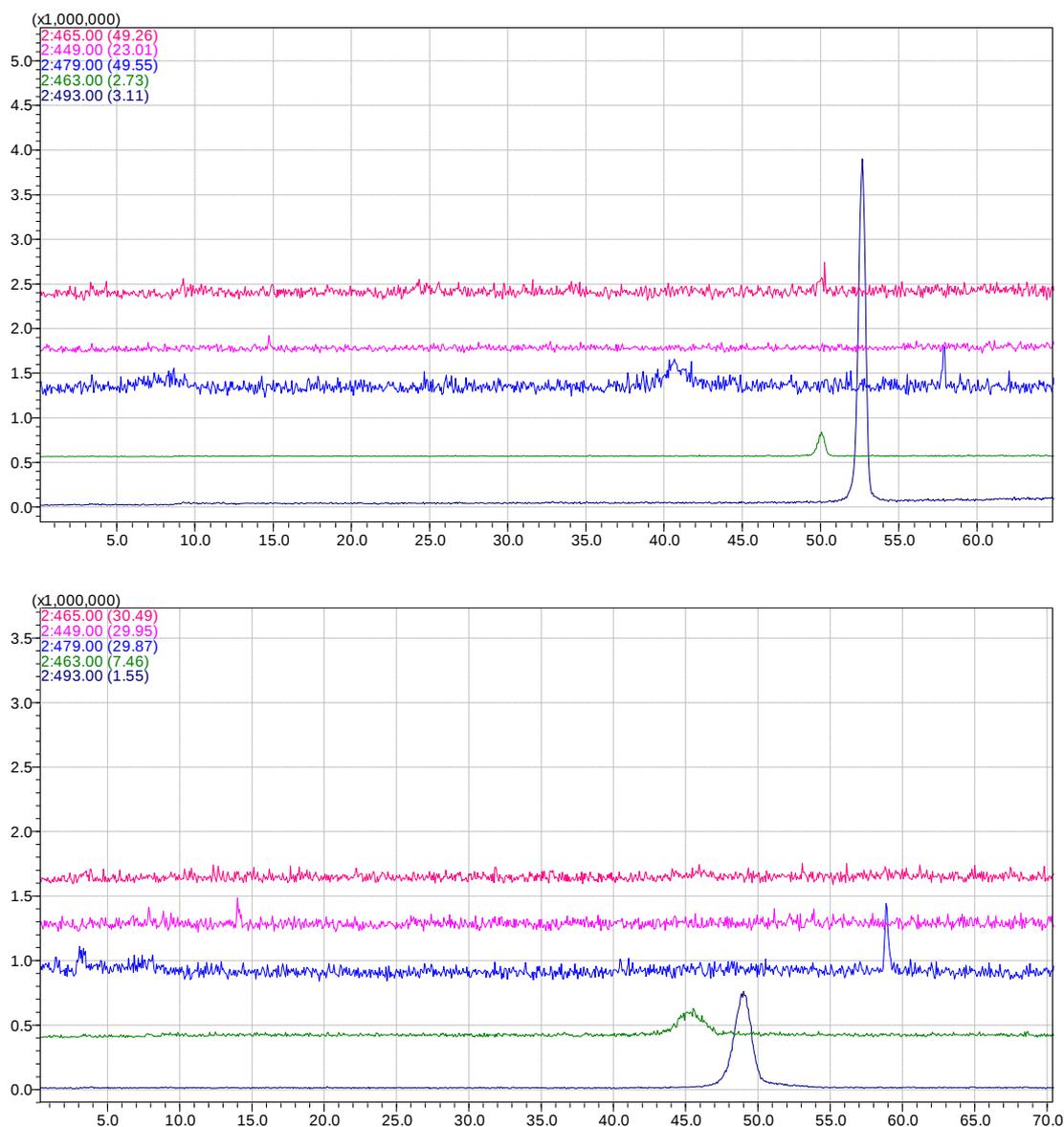


Fig. 43: R23, R24 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) vs Mv-3-Glu ($m/z = 493$); 1%, 0.1% B respectively:

Separation factor (α): 1.06, 1.08

Resolution (R_s): 5.20, 2.23

There is a clear loss of separation and resolution upon reducing the formic acid concentration to 1 or 0.1%. Although 1% formic acid resolves Pn-3-Glu and Mv-3-Glu with an acceptable R_s of 5.20, the Pt-3-Glu signal is too noisy to be quantified and Dl-3-Glu is completely lost. This is especially clear when comparing these two chromatograms with R21 (10% formic acid), in which Dl-3-Glu and Pt-3-Glu are both clearly visible. This is presumably due to reduced flavylum concentrations in the less acidified solutions. The conclusion is that 10% formic acid is required for the separation and identification of anthocyanins, at least for samples of this strength.

3.22 Faster solvent B gradient, stronger sample (R25)

The final iteration of the anthocyanin LC/MS method development focused on reducing the time required for analysis of the five main anthocyanins. To this end only one gradient was used (i.e. no attempt made to separate late-eluting compounds) and the rate was increased to 0.33% B/minute. The 100% and 0% B column conditioning stages were also reduced from 10 to 8 minutes. The method was applied to the full strength sample of Pinot Noir, obtained directly from fractionation without dilution. All other conditions were identical to R20.

Sample: Pinot Noir, full strength

Injection volume: 2 μ L

Flow rate: 0.2 mL/min

Oven temperature: 50 $^{\circ}$ C

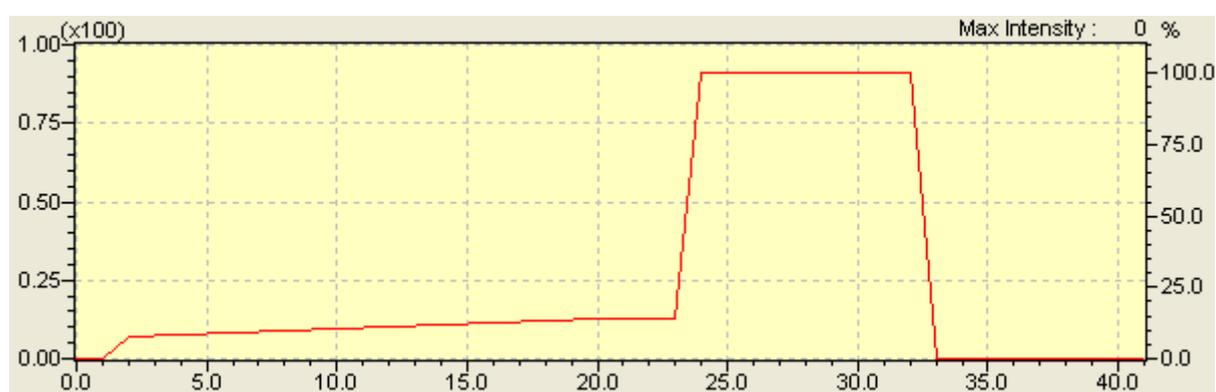
Acid: Formic

Acid strength: 10% (vol/vol)

Gradient(s): 8-14,14(isocratic)% B

Method file: asf1013_2.lcm

Data file: asf1013_2.lcd



	Time	Module	Action	Value
1	1.00	Pumps	B.Conc	0
2	2.00	Pumps	B.Conc	8
3	20.00	Pumps	B.Conc	14
4	23.00	Pumps	B.Conc	14
5	24.00	Pumps	B.Conc	100
6	32.00	Pumps	B.Conc	100
7	33.00	Pumps	B.Conc	0
8	41.00	Pumps	B.Conc	0
9	41.00	Controller	Stop	

Fig. 44: R25 solvent B gradient, LC program

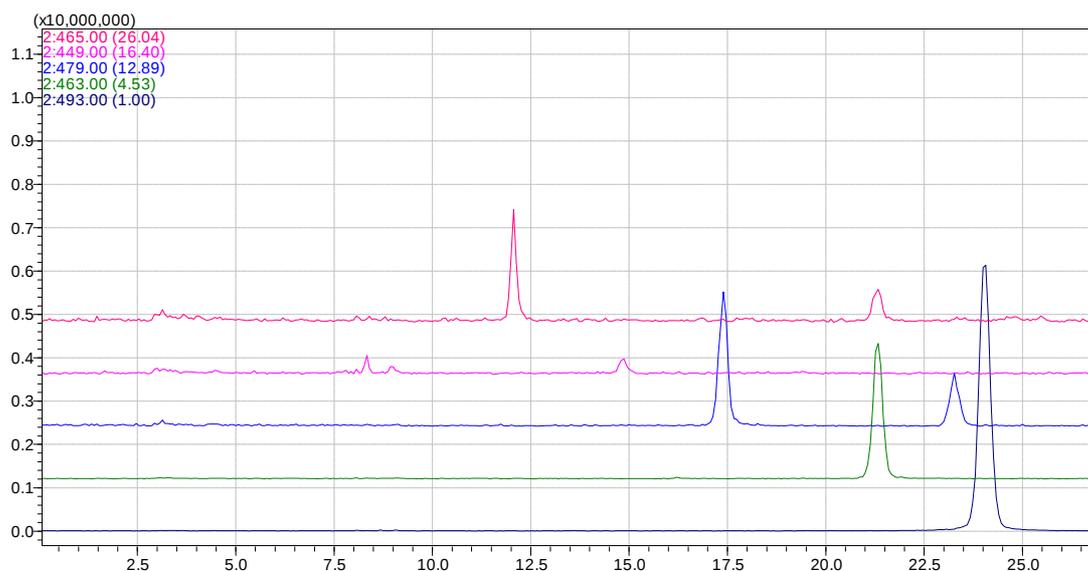


Fig. 45: R25 MS SIM anthocyanin chromatograms

Pn-3-Glu ($m/z = 463$) vs Mv-3-Glu ($m/z = 493$):

Separation factor (α): 1.15

Resolution (R_s): 9.41

The increase in solvent B gradient slope reduces the resolution from that observed in R20 (14.40) to 9.41, which is still quite adequate for baseline separation of the main anthocyanins. The increased sample strength is evident in $m/z = 449$ channel which clearly shows a small amount of Cy-3-Glu present in the wine sample. We also see two peaks in the $m/z = 465$ and 479 channels, corresponding to two types of delphinidin- and petunidin-containing molecules. The larger peaks ($m/z = 465$ $t_R = ca. 12$ min, $m/z = 479$ $t_R = ca. 17.5$ min), which follow the expected elution order, are the respective 3-*O*-glucosides, visible in the 520 nm PDA spectrum (Fig. 46, pink trace), as expected.

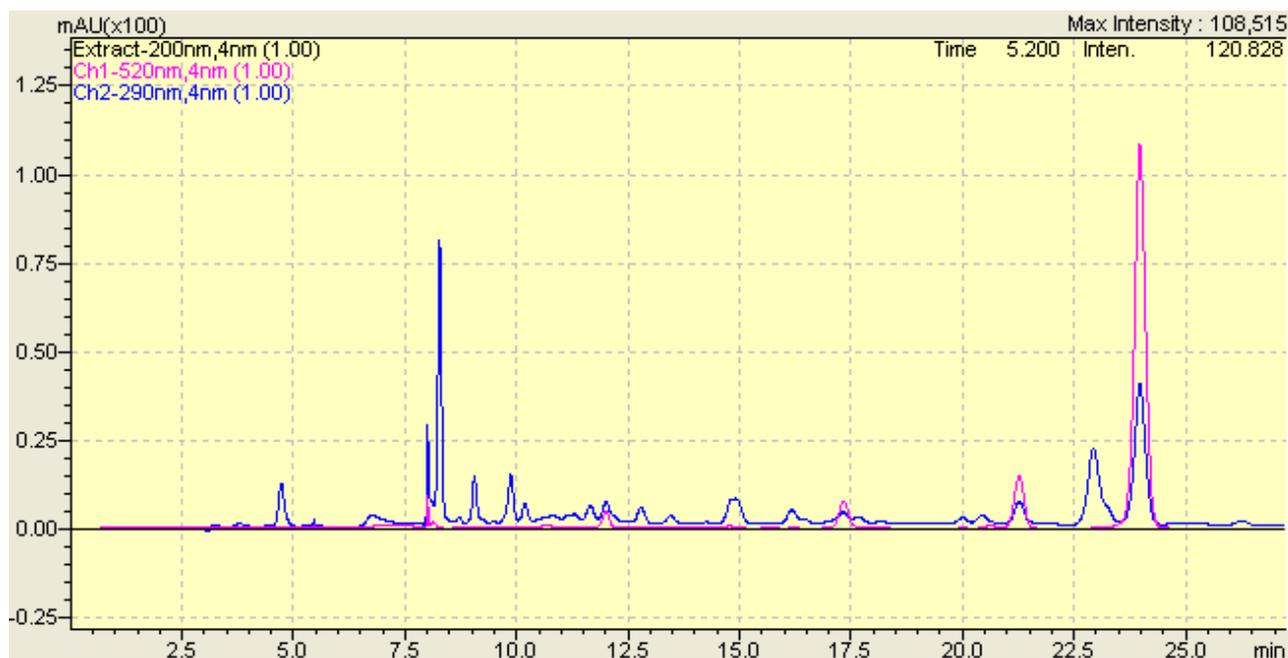


Fig. 46: R25 290nm (blue) and 520 nm (pink) chromatograms

The minor peak in the $m/z = 479$ channel ($t_R = \text{ca. } 23 \text{ min}$) is seen as an absorbance in the 290 nm PDA spectrum but not in the 520 nm spectrum, implying that the peak is not coloured, thus ruling out any flavylum forms of petunidin (such as petunidin-3-*O*-galactoside, which has a molecular mass identical to the glucoside but would also be expected to absorb at 520 nm). Since the minor peak in the $m/z = 465$ channel ($t_R = \text{ca. } 21 \text{ min}$) co-elutes with Pn-3-Glu we cannot determine whether it is coloured or not. Using 520 nm PDA detection will prevent the minor peak in the $m/z = 479$ channel interfering with the quantitation of Mv-3-Glu, whilst MS detection in SIM mode will allow Pn-3-Glu to be quantitated separately from the minor peak in the $m/z = 465$ channel.

The fast solvent B gradient used in R25 successfully reduced the time required for Mv-3-Glu to elute to 25 min, compared with 34 min (R22) and 43 min (R20) using slower gradients. The total time required for R25, from sample injection to the end of column conditioning, is 41 min.

4 Results and Discussion - Late-eluting pigment method development

The second aim of the project was to examine the effect of different LC/MS methods on the separation and identification of late-eluting pigments, such as pyranoanthocyanins and ethyl-bridged dimers. Because of the large number of potential late-eluting species, development was initially conducted by examining PDA chromatograms, with the much more complicated MS SIM chromatograms subsequently used for identifying the PDA peaks.

The solvent B gradient required for anthocyanin separation using the optimised conditions developed previously (10% formic acid, 50 °C oven temperature, 0.2 mL/min column flow rate) was in the region of 8-11% B (e.g. R20). This gradient was extended to higher %B values to allow late-eluting compounds to be separated.

4.1 Extending B gradient to 30% (R21)

R21 was previously analysed as part of the anthocyanin method development, beginning with the same 0.05%B/min slope used in R20 up to the point at which Mv-3-Glu eluted (9.75% B). From this point a steeper gradient was used to see if the later-eluting compounds could be separated as part of the same LC method. All other conditions were identical to R20.

Sample: Pinot Noir, 10:1 dilution in methanol

Injection volume: 2 μ L

Flow rate: 0.2 mL/min

Oven temperature: 50 $^{\circ}$ C

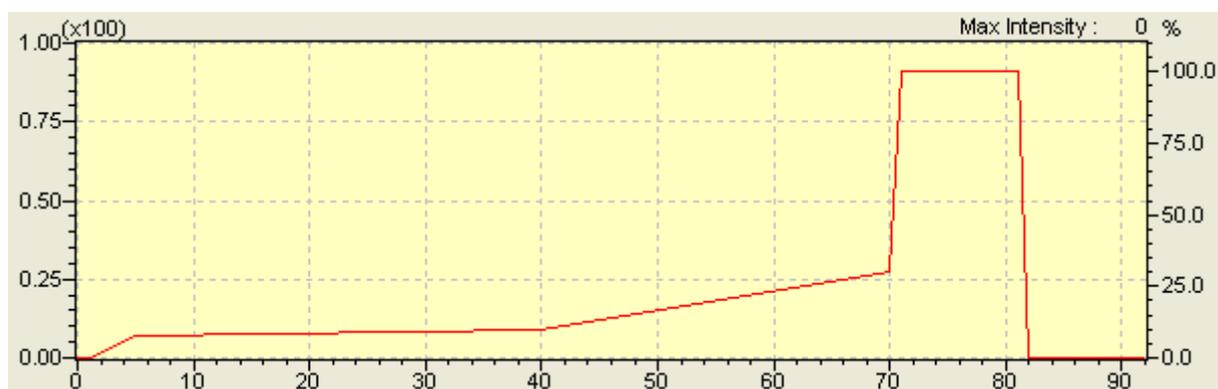
Acid: Formic

Acid strength: 10% (vol/vol)

Gradient(s): 8-9.75-30% B

Method file: asf907_2.lcm

Data file: asf907_2.lcd



	Time	Module	Action	Value
1	1.00	Pumps	B.Conc	0
2	5.00	Pumps	B.Conc	8
3	40.00	Pumps	B.Conc	9.75
4	70.00	Pumps	B.Conc	30
5	71.00	Pumps	B.Conc	100
6	81.00	Pumps	B.Conc	100
7	82.00	Pumps	B.Conc	0
8	92.00	Controller	Stop	

Fig. 47: R21 solvent B gradient, LC program



Fig. 48: R21 520 nm PDA chromatogram (Mv-3-Glu at ca. 41 min)

The 9.75-30% B gradient separated some late-eluting pigments, visible as small absorbances after Malv, the last of the anthocyanins to elute. However, these pigments were eluting quite close to the end of the gradient, suggesting a further extension to the B gradient was required to rule out even later eluting compounds.

4.2 Extending B gradient to 100% (R22)

This method was also used as part of the anthocyanin method development. The second gradient in run R21 was extended to 100% B to ensure all possible late-eluting compounds would be separated by the method. All other conditions were identical to R20. A faster 8-10% B initial gradient also helped reduce total analysis time.

Sample: Pinot Noir, 10:1 dilution in methanol

Injection volume: 2 μ L

Flow rate: 0.2 mL/min

Oven temperature: 50 $^{\circ}$ C

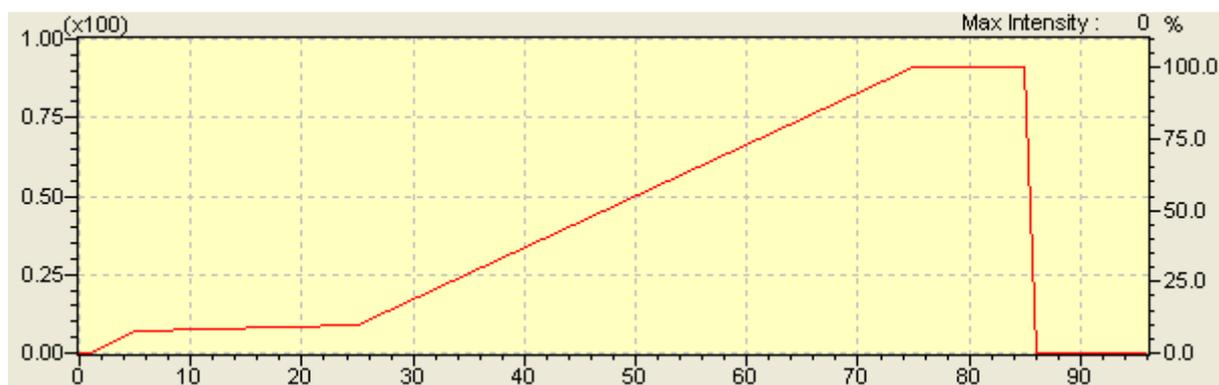
Acid: Formic

Acid strength: 10% (vol/vol)

Gradient(s): 8-10-100% B

Method file: asf907_3.lcm

Data file: asf907_3.lcd



	Time	Module	Action	Value
1	1.00	Pumps	B.Conc	0
2	5.00	Pumps	B.Conc	8
3	25.00	Pumps	B.Conc	10
4	75.00	Pumps	B.Conc	100
5	85.00	Pumps	B.Conc	100
6	86.00	Pumps	B.Conc	0
7	96.00	Controller	Stop	

Fig. 49: R22 solvent B gradient, LC program



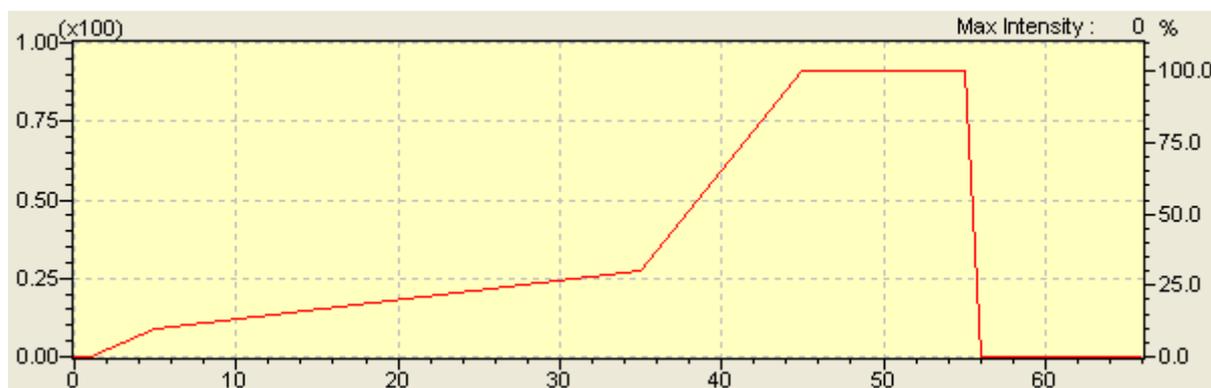
Fig. 50: R22 520 nm PDA chromatogram (Mv-3-Glu at ca. 33 min)

The second 10-100% B gradient proved successful at separating some late-eluting pigments ($t_R = 35 - 38$ min, also possibly some after 43 min), but the strength of the sample clearly needed increasing to help detection.

4.3 Stronger sample, 0.1% formic acid (R26)

A stronger sample was used to increase the signal-to-noise of the late-eluting pigments. The MS detector was expected to recover in time for detection of the late-eluting pigments, should saturation occur during elution of the anthocyanins. The formic acid concentration was reduced to 0.1% to aid MS detection of the late-eluting pigments. The effect of low acid concentration on late-eluting peak shapes was also of interest.

Sample: Pinot Noir, full strength
 Injection volume: 2 μ L
 Flow rate: 0.2 mL/min
 Oven temperature: 50 $^{\circ}$ C
 Acid: Formic
 Acid strength: 0.1% (vol/vol)
 Gradient(s): 9.5-30-100% B
 Method file: asf910_1.lcm
 Data file: asf910_1.lcd



	Time	Module	Action	Value
1	1.00	Pumps	B.Conc	0
2	5.00	Pumps	B.Conc	9.5
3	35.00	Pumps	B.Conc	30
4	45.00	Pumps	B.Conc	100
5	55.00	Pumps	B.Conc	100
6	56.00	Pumps	B.Conc	0
7	66.00	Pumps	B.Conc	0
8	66.00	Controller	Stop	

Fig. 51: R26 solvent B gradient, LC program



Fig. 52: R26, 520 nm PDA chromatogram (Mv-3-Glu at ca. 19 min)
SIM channels marked (see Table 4), n = no channel match, unknown

The latest eluting peaks, at $t_R =$ ca. 35.5 and 36.5 min, indicate under these conditions the B gradient only needs to run up to 30%. The increased sample strength is evident in the less noisy baseline. The lower acid concentration resulted in poor peak shapes, with some tailing visible, however, since quantitation is not required, this is not necessarily a problem.

The MS SIM channels for this run were selected by reference to a comprehensive list of late-eluting pigments previously reported in Californian Cabernet Sauvignon wines and grape skin extracts by Wang (2004). This resulted in a total of 29 channels, analysed in two sections for convenience. The first section (channels 1-11, Table 2) contains various anthocyanin-3-*O*-acylglucosides (R26 MS chromatograms: Fig. 53), and the second section (channels 12-29, Table 3) contains pyranoanthocyanins, ethyl-bridged dimers and other pigments (R26 chromatograms: Fig. 54). Within each section the compounds are listed in their elution order under the particular conditions employed in Wang's study (Solvent A: 10% acetic acid, 0.1% trifluoroacetic acid, 5% acetonitrile, remainder water; Solvent B: acetonitrile; column oven temperature 25 °C; solvent B gradient: 0-10% (0-30 min) 10-30% B (30-40 min) 30-40% B (40-50 min); flow rate 0.2 mL/min; 150 x 2.0 mm C₁₈ RP column). Using an existing list of compounds as the basis for multi-channel SIM detection allowed for the quick identification of the majority of late-eluting compounds in R26.

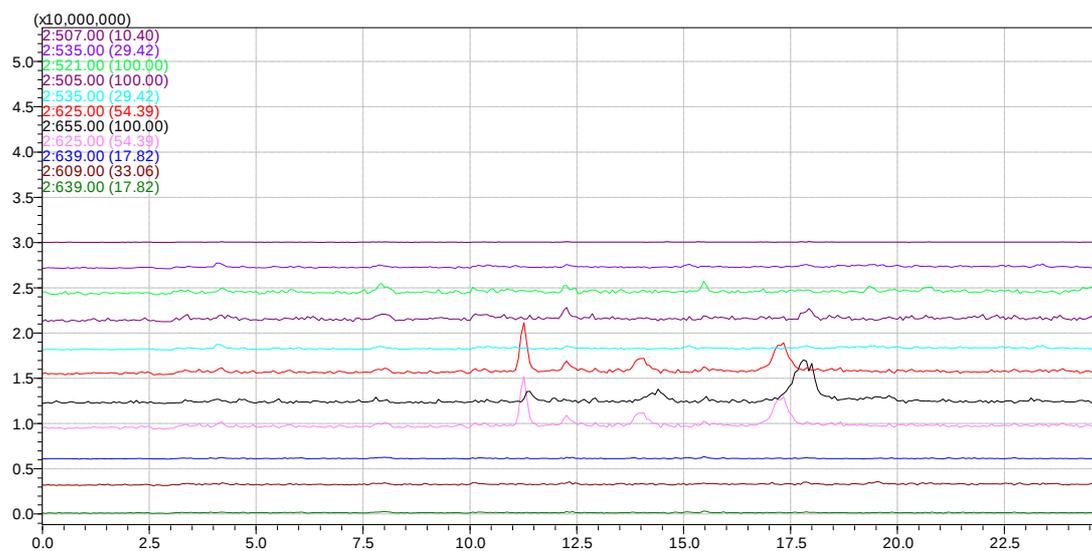


Fig. 53: R26 SIM channel 1-11 chromatograms (acylglucosides)

Channel	<i>m/z</i>	Compound
1	507	delphinidin-3- <i>O</i> -acetylglucoside
2	535	malvidin-3- <i>O</i> -acetylgalactoside
3	521	petunidin-3- <i>O</i> -acetylglucoside
4	505	peonidin-3- <i>O</i> -acetylglucoside
5	535	malvidin-3- <i>O</i> -acetylglucoside
6	625	peonidin-3- <i>O</i> -caffeoylglucoside
7	655	malvidin-3- <i>O</i> -caffeoylglucoside
8	625	petunidin-3- <i>O</i> -coumaroylglucoside
9	639	malvidin-3- <i>O</i> -coumaroylgalactoside
10	609	peonidin-3- <i>O</i> -coumaroylglucoside
11	639	malvidin-3- <i>O</i> -coumaroylglucoside

Table 2: MS SIM channels 1-11 (acylglucosides)

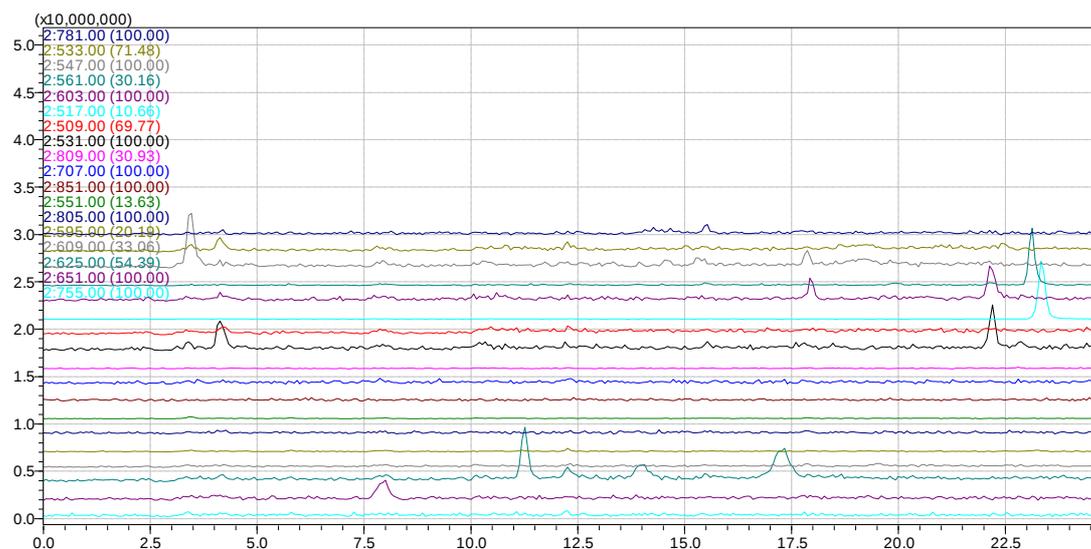


Fig. 54: R26, SIM channel 12-29 chromatograms (pyranoanthocyanins, etc.)

Channel	<i>m/z</i>	Compound
12	781	malvidin-3- <i>O</i> -glucoside-(<i>epi</i>)-catechin
13	533	5-carboxyl-pyranodelphinidin-3- <i>O</i> -glucoside
14	547	5-carboxyl-pyranopetunidin-3- <i>O</i> -glucoside
15	561	5-carboxyl-pyranomalvidin-3- <i>O</i> -glucoside (vitisin A)
16	603	5-carboxyl-pyranomalvidin-3- <i>O</i> -acetylglucoside
17	517	pyranomalvidin-3- <i>O</i> -glucoside
18	509	syringetin-3- <i>O</i> -glucoside
19	531	5-methyl-pyranomalvidin-3- <i>O</i> -glucoside
20	809	malvidin-3- <i>O</i> -glucoside-ethyl-(<i>epi</i>)-catechin
21	707	malvidin-3- <i>O</i> -coumaroylglucoside-pyruvate
22	851	malvidin-3- <i>O</i> -acetylglucoside-ethylflavanol
23	551	syringetin-3- <i>O</i> -acetylglucoside
24	805	malvidin-3- <i>O</i> -glucoside-4-vinyl-(<i>epi</i>)-catechin
25	595	5-(4'-hydroxyphenyl)-pyranopetunidin-3- <i>O</i> -glucoside
26	609	5-(4'-hydroxyphenyl)-pyranomalvidin-3- <i>O</i> -glucoside
27	625	5-(3',4'-dihydroxyphenyl)-pyranomalvidin-3- <i>O</i> -glucoside
28	651	5-(4'-hydroxyphenyl)-pyranomalvidin-3- <i>O</i> -acetylglucoside
29	755	5-(4'-hydroxyphenyl)-pyranomalvidin-3- <i>O</i> -coumaroylglucoside

Table 3: MS SIM channels 12-29 (pyranoanthocyanins, etc.)

By correlating 520nm PDA absorbances with SIM MS peaks, the possible identities of most of the late-eluting peaks could be determined (Table 4). It is important to note that in the absence of other data, an ESI MS m/z match can only be taken as a provisional form of characterisation. Matching the UV-Vis spectrum of each peak in the PDA chromatogram against any published UV-Vis spectra of the proposed compound might provide additional confirmation, however the UV-Vis spectra of many anthocyanin-based pigments are pH dependent, meaning separate HPLC runs using solvents adjusted to the published pH values could be required for each peak of interest. Due to time constraints this study only presents tentative characterisation based on SIM MS data.

Table 4 presents the MS-based identification of peaks in R26, based on correlation between the 520nm PDA and MS SIM channels 1-29. In cases where a given m/z ratio corresponds to more than one compound, both are listed.

t_R (min)	Channel	m/z	Compound(s)
22.262	16	603	5-carboxyl-pyranomalvidin-3- <i>O</i> -acetylglucoside
23.102	15	561	5-carboxyl-pyranomalvidin-3- <i>O</i> -glucoside (vitisin A)
23.312	17	517	pyranomalvidin-3- <i>O</i> -glucoside
24.993	20	809	malvidin-3- <i>O</i> -glucoside-ethyl-(epi)-catechin (or isomer)
25.553			n/m
25.833	20	809	malvidin-3- <i>O</i> -glucoside-ethyl-(epi)-catechin (or isomer)
26.253	20	809	malvidin-3- <i>O</i> -glucoside-ethyl-(epi)-catechin (or isomer)
27.268	20	809	malvidin-3- <i>O</i> -glucoside-ethyl-(epi)-catechin (or isomer)
35.634	10	609	peonidin-3- <i>O</i> -coumaroylglucoside OR
	26	609	5-(4'-hydroxyphenyl)-pyranomalvidin-3- <i>O</i> -glucoside
36.790	11	639	malvidin-3- <i>O</i> -coumaroylglucoside OR
	9	639	malvidin-3- <i>O</i> -coumaroylgalactoside

Table 4: R26, retention times and SIM MS-identified compounds (n/m: no match, unidentified)

Based purely on MS m/z ratios it is not possible to exclusively assign the peaks at $t_R = 35.634$ and 36.790 min. It is interesting to note that acylglycosides are normally thought to be totally absent in Pinot Noir grapes and wines (Revilla 2012). Assuming their presence in this sample is not due to winemaking decisions (e.g. the inclusion of non-Pinot Noir fruit in the fermentation), or cross-contamination during processing, these peaks might warrant more thorough investigation. Similarly, conclusive identification of the peak at $t_R = 22.262$, assigned as 5-carboxyl-pyranomalvidin-3-*O*-

acetylglucoside, and confirmation that it is indeed derived from Pinot Noir, would also be of interest.

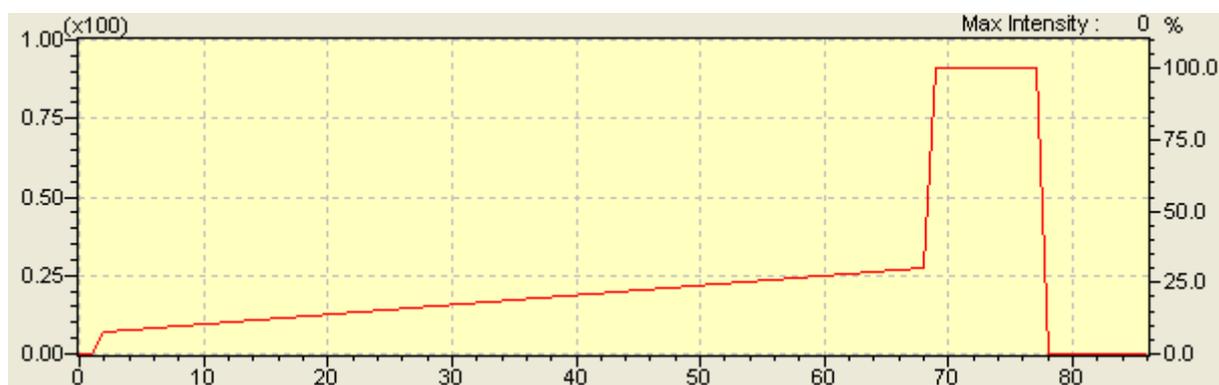
The four peaks at $t_R = 24.993$, 25.833 , 26.253 and 27.268 min each correspond to $m/z = 809$, which is assigned in Wang's publication (2004) to malvidin-3-*O*-glucoside-ethyl-(epi)-catechin. Ethyl-bridged dimers form from the reaction of a flavan-3-ol with a molecule of electrophilic, protonated acetaldehyde followed by reaction with an anthocyanin hemiketal. The presence of four separate peaks can be rationalised by the reaction of both (+)-catechin and (-)-(epi)-catechin with protonated acetaldehyde, followed by reaction at either the 8- or 6-positions of Mv-3-Glu.

The remaining peaks $t_R = 23.102$ and 23.312 min were identified as 5-carboxyl-pyranomalvidin-3-*O*-glucoside (vitisin A) and pyranomalvidin-3-*O*-glucoside, respectively. The peak at $t_R = 25.553$ does not correspond to any of the SIM channels

5 Results and Discussion - Church Road LC/MS method

An LC/MS run was carried out on each of four fractionated samples of Church Road wines vinified from Cabernet Sauvignon, Malbec, Merlot and Syrah varieties. The fractionating was carried out by the same method used for the previous samples (Jeffery 2008), and the first fractions were used without dilution. The method used for these runs (asf1013_4.lcm) was designed to allow anthocyanin and later-eluting pigments to be separated using one single LC/MS analysis, by taking the same solvent B starting concentration and gradient used to separate anthocyanins in R25 (starting B concentration 8%, gradient 0.333% B per minute) and extending it to 30% B, allowing all late-eluting pigments to elute (as determined in R26).

Injection volume: 2 μ L
 Flow rate: 0.2 mL/min
 Oven temperature: 50 °C
 Gradient(s): 8-30% B
 Method file: asf1013_4.lcm



	Time	Module	Action	Value
1	1.00	Pumps	B.Conc	0
2	2.00	Pumps	B.Conc	8
3	68.00	Pumps	B.Conc	30
4	69.00	Pumps	B.Conc	100
5	77.00	Pumps	B.Conc	100
6	78.00	Pumps	B.Conc	0
7	86.00	Pumps	B.Conc	0
8	86.00	Controller	Stop	

Fig. 55: R27-R32 solvent B gradient, LC program

The four Church Road #1 fractions were analysed using method asf1013_4.lcm, with 10% formic acid (Table 5). The MS SIM anthocyanin chromatograms of these runs R27 - R30 were manually integrated to give the relative proportions of anthocyanins in each wine (Section 5.1)

The Cabernet Sauvignon fraction #1 chromatogram R27 was also studied in terms of late-eluting pigment content (see section 5.2). Cabernet Sauvignon was chosen since it is the same varietal used in the comprehensive late-eluting pigment analysis presented by Wang (2004). In addition, two extra runs were carried out on the Cabernet Sauvignon fraction #1 using 1% (R31) and 0.1% formic acid (R32), to see if lower acid strengths assisted the MS detection of late-eluting compounds.

Varietal (fraction #1)	Run #	Formic acid (v/v)
Cabernet Sauvignon	R27	10%
Malbec	R28	10%
Merlot	R29	10%
Syrah	R30	10%
Cabernet Sauvignon	R31	1%
Cabernet Sauvignon	R32	0.1%

Table 5: Church Road wine runs

5.1 Church Road Varietals - Anthocyanin analyses

The anthocyanin MS SIM chromatogram obtained for Cabernet Sauvignon (Fig. 56) is representative of those obtained for the other three varietals (data not shown).

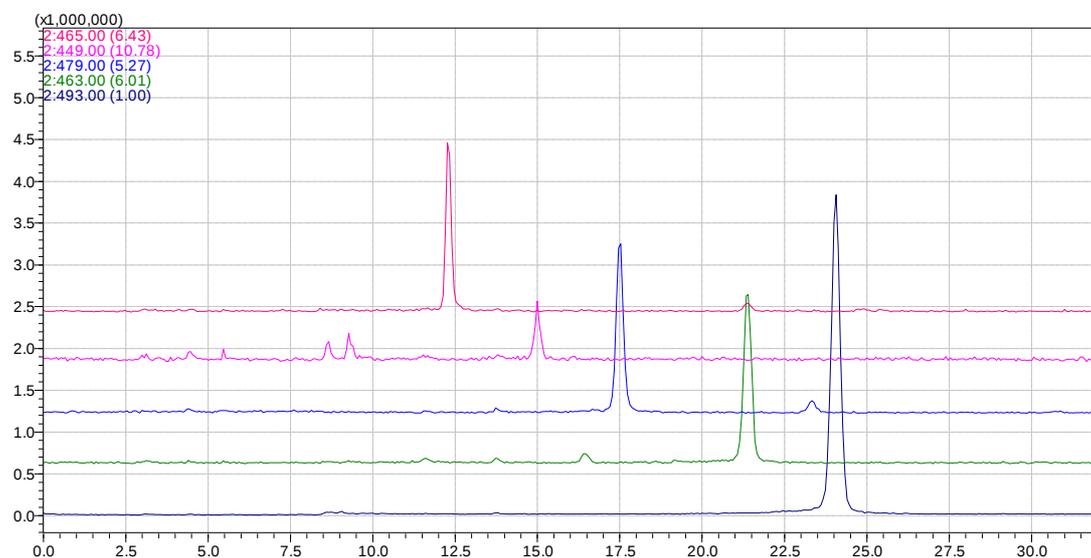


Fig. 56: R27, Church Road Cabernet Sauvignon MS SIM anthocyanin chromatograms

Manual integration of the SIM chromatograms provided peak areas (in arbitrary units) and the percent composition of the five anthocyanins, presented in Table 6 for each of the four varietals.

	Cabernet Sauv. (R27)	Malbec (R28)	Merlot (R29)	Syrah (R30)
DI-3-Glu	3675106 (4.12%)	3676948 (2.57%)	2136488 (3.81%)	2588152 (2.79%)
Cy-3-Glu	723638 (0.81%)	544612 (0.38%)	637204 (1.14%)	524620 (0.57%)
Pt-3-Glu	5885628 (6.60%)	9319002 (6.50%)	3846232 (6.87%)	6353062 (6.84%)
Pn-3-Glu	5682962 (6.37%)	6974736 (4.87%)	4444430 (7.94%)	8720676 (9.39%)
Mv-3-Glu	73229900 (82.1%)	122830054 (85.7%)	55957990 (80.2%)	92840830 (80.4%)
Total (100%)	89197234	143345352	55957990	92840830

Table 6: Church Road varietals, anthocyanin peak areas (arbitrary units)

Since the four samples were fractionated and analysed identically, the total anthocyanins can be compared, revealing a significant variation with variety. Relative to Malbec, the total anthocyanin-3-*O*-glucoside contents of the other varietal wines were 65% (Syrah), 62% (Cabernet Sauvignon) and 39% (Merlot). Conversely, the proportions of anthocyanins follow a similar pattern between the four varieties, with malvidin-3-*O*-glucoside the most prevalent anthocyanin, followed by petunidin-3-*O*-glucoside \approx peonidin-3-*O*-glucoside, then delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside as the smallest contributor. The proportions of anthocyanin-3-*O*-glucosides in each of the four varieties are compared with literature results in Tables 7 - 10.

	R27	Gutierrez (2005)	Dimitrovska (2011)	Ginjom (2011)
Sample type	W (4)	W (0)	S	W (0)
Dl-3-Glu	4.12	8.59	6.89	6.31
Cy-3-Glu	0.81	0.20	1.24	n/r
Pt-3-Glu	6.60	8.76	7.39	6.31
Pn-3-Glu	6.37	4.00	5.92	8.13
Mv-3-Glu	82.1	78.5	78.6	79.2

Table 7: Cabernet Sauvignon, anthocyanin-3-*O*-glucosides (%)
(W = wine (months after fermentation), S = grape skin, n/r = not reported)

	R28	Fanzone (2010)	Antoniolli (2015)
Sample type	W (4)	W (0)	P
Dl-3-Glu	2.57	8.5	8
Cy-3-Glu	0.38	3.5	4
Pt-3-Glu	6.50	10.6	16
Pn-3-Glu	4.87	4.5	9
Mv-3-Glu	85.7	72.9	63

Table 8: Malbec, anthocyanin-3-*O*-glucosides (%)
(W = wine (months after fermentation), P = grape pomace, n/r = not reported)

	R29	Dimitrovska (2011)	Girelli (2015)
Sample type	W (4)	S	W (n/r)
Dl-3-Glu	3.81	15.4	18
Cy-3-Glu	1.14	3.13	27
Pt-3-Glu	6.87	13.0	7
Pn-3-Glu	7.94	7.70	6
Mv-3-Glu	80.2	60.7	42

Table 9: Merlot, anthocyanin-3-*O*-glucosides (%)

(W = wine (months after fermentation), S = grape skins, n/r = not reported)

	R30	Gutierrez (2005)	Ginjom* (2011)	Girelli (2015)
Sample type	W (4)	W (0)	S	W (n/r)
Dl-3-Glu	2.79	4.90	4.0 - 5.3	18
Cy-3-Glu	0.57	0.52	2.0 - 3.2	22
Pt-3-Glu	6.84	9.39	7.4 - 8.5	5
Pn-3-Glu	9.39	4.44	17 - 22	6
Mv-3-Glu	80.4	80.8	61 - 69	49

Table 10: Shiraz/Syrah, anthocyanin-3-*O*-glucosides (%)

(W = wine (months after fermentation), S = grape skin, n/r = not reported,

*range for berries at 23°B, 25°B and 28°B ripeness)

The proportion of anthocyanin-3-*O*-glucosides found in Church Road Cabernet Sauvignon (R27) is broadly similar to those previously reported for young wines and grape skin extracts in the literature. In the Church Road Malbec sample (R28) Dl-3-Glu, Cy-3-Glu and Pt-3-Glu appear to be under-represented, whilst Mv-3-Glu is present in larger amounts. Dl-3-Glu and Cy-3-Glu also appear to be under-represented and Mv-3-Glu over-represented in the Church Road Merlot wine (R29). The values found for Church Road Syrah (R30) are quite similar to those reported by Gutierrez (2005), however there is wide variation in the literature values. It is worth noting that the

Merlot and Shiraz percentages found by Girelli (2015) were obtained by analysis of bottled, commercial wines of unspecified ages. Thus the proportions of anthocyanin-3-*O*-glucosides may have been significantly altered due to selective consumption by subsequent reactions such as those which form pyranoanthocyanins and polymeric pigments in the bottle. Differences in extractive and analytical techniques might be an important factor leading to the sometimes significant variations between literature sources.

The results of the Church Road wine analyses demonstrate the utility of the LC/MS method development as applied to the separation of anthocyanin-3-*O*-glucoside pigments, which are the most important contributors of colour in young red wines.

5.2 Church Road Cabernet Sauvignon - Late-eluting pigment analysis

The Church Road Cabernet Sauvignon fraction #1 (R27, using 10% formic acid) was also analysed in terms of late-eluting pigments, such as anthocyanin-3-*O*-acylglycosides, pyranoanthocyanins and polymeric pigments. The 520 nm PDA chromatogram is shown in Fig. 57.

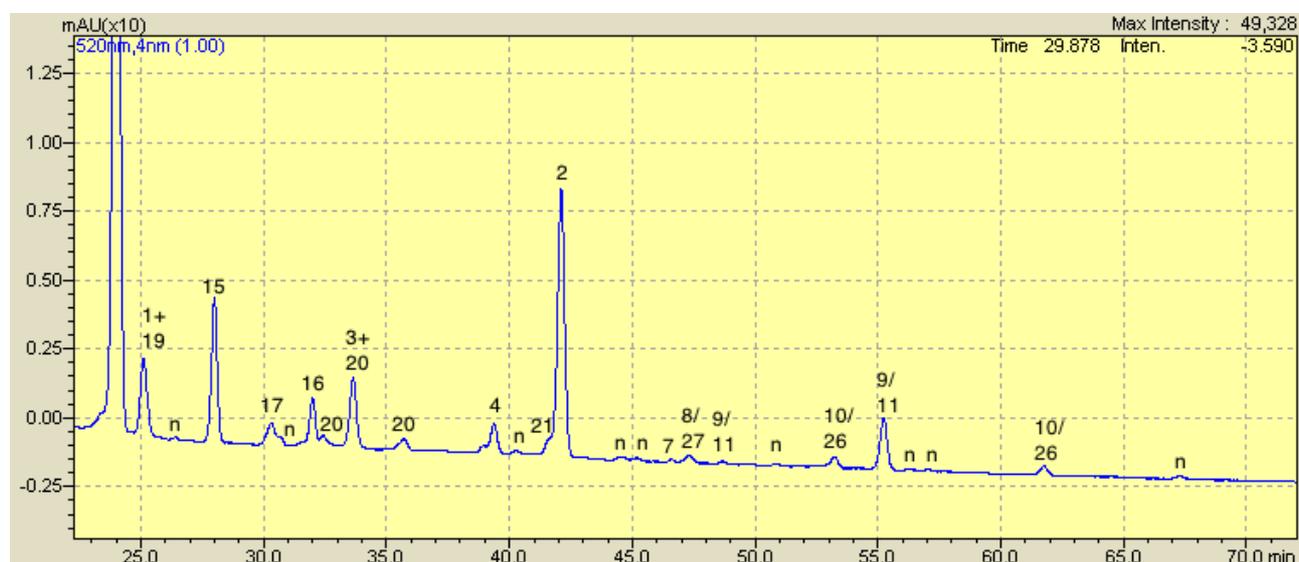


Fig. 57: R27, 520 nm PDA chromatogram (Mv-3-Glu at ca. 24 min)
SIM channels marked (see Table 11), n = no channel match, unknown

Similarly to the late-eluting pigment LC/MS method development (R26, Section 4.3), analysis was carried out by selecting 29 MS SIM channels which corresponded to the *m/z* values of late-eluting pigments previously reported by Wang (2004) in Californian Cabernet Sauvignon grapes and wines. The 29 SIM channels are described in Tables 2 and 3, and the peak assignments in R27 are listed in Table 11.

t_R (min)	Channel	m/z	Compound(s)
25.133	1	507	delphinidin-3- <i>O</i> -acetylglucoside AND
	19	531	5-methyl-pyranomalvidin-3- <i>O</i> -glucoside
26.337		n/m	
28.010	15	561	5-carboxyl-pyranomalvidin-3- <i>O</i> -glucoside (vitisin A)
30.351	17	517	pyranomalvidin-3- <i>O</i> -glucoside
30.753		n/m	
32.024	16	603	5-carboxyl-pyranomalvidin-3- <i>O</i> -acetylglucoside
32.492	20	809	malvidin-3- <i>O</i> -glucoside-ethyl-(epi)-catechin (or isomer)
33.629	3	521	petunidin-3- <i>O</i> -acetylglucoside AND
	20	809	malvidin-3- <i>O</i> -glucoside-ethyl-(epi)-catechin (or isomer)
35.637	20	809	malvidin-3- <i>O</i> -glucoside-ethyl-(epi)-catechin (or isomer)
39.316	4	505	peonidin-3- <i>O</i> -acetylglucoside
40.253		n/m	
41.534	21	707	5-carboxyl-pyranomalvidin-3- <i>O</i> -acetylglucoside
42.059	2	535	malvidin-3- <i>O</i> -acetylgalactoside
44.535		n/m	
45.204		n/m	
46.609	7	655	malvidin-3- <i>O</i> -caffeoylglucoside
47.412	8	625	petunidin-3- <i>O</i> -coumaroylglucoside OR
	27		5-(3',4'-dihydroxyphenyl)-pyranomalvidin-3- <i>O</i> -glucoside
48.683	11	639	malvidin-3- <i>O</i> -coumaroylglucoside OR
	9		malvidin-3- <i>O</i> -coumaroylgalactoside
50.824		n/m	
53.232	10	609	peonidin-3- <i>O</i> -coumaroylglucoside OR
	26		5-(4'-hydroxyphenyl)-pyranomalvidin-3- <i>O</i> -glucoside
55.239	11	639	malvidin-3- <i>O</i> -coumaroylglucoside OR
	9		malvidin-3- <i>O</i> -coumaroylgalactoside
56.310		n/m	
56.912		n/m	
61.729	10	609	peonidin-3- <i>O</i> -coumaroylglucoside OR
	26		5-(4'-hydroxyphenyl)-pyranomalvidin-3- <i>O</i> -glucoside
67.349		n/m	

Table 11: R27, retention times and SIM MS-identified compounds (n/m: no match, unidentified)

Of the compounds reported by Wang (2004), a large number have been tentatively identified in the Church Road Cabernet Sauvignon sample. The peaks in the PDA chromatogram which did not correlate to a SIM channel were all very small, suggesting that an increase in analyte quantity might be required to allow MS detection of these trace components. This could be carried out by using injection volumes $>2 \mu\text{L}$, or by preparing more concentrated fractions during the initial solid phase sample extraction stage. Stronger samples would run the risk of saturating the detector during the elution of the anthocyanin-3-*O*-glucoside peaks, thus requiring a separate run to analyse these much stronger components. As performed in this study, R27 is a demonstration of a single-injection LC/MS analysis which was able to quantify the 5 main anthocyanin-3-*O*-glucosides, and in addition, tentatively identify a total of 14 late-eluting acylglycosides, pyranoanthocyanins and ethyl-bridged dimers.

The final stage of the Church Road Cabernet Sauvignon late-eluting pigment analysis was to examine the effect of lower acid concentrations on chromatogram peak shape and MS detection sensitivity. Two additional runs were performed using conditions identical to R27 except with 1% formic acid (R31) and 0.1% formic acid (R32) in place of the 10% formic acid used in R27. The PDA chromatograms are shown in Fig. 58 and 59.

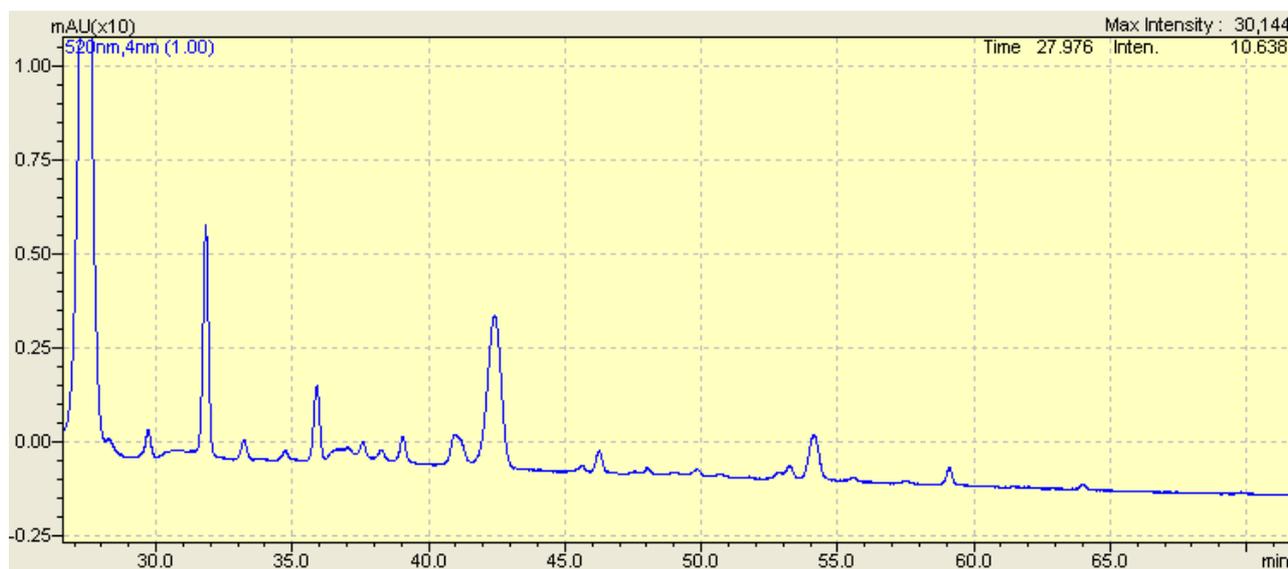


Fig 58: R31, 520 nm PDA chromatogram (Mv-3-Glu at ca. 27 min), 1% formic acid



Fig 59: R32, 520 nm chromatogram (Mv-3-Glu at ca. 24 min), 0.1% formic acid

Compared with R27, the reduction in solvent acidity caused a broadening of peak widths, especially in R32. However, MS SIM detection was still largely successful in the less-acidic runs. Compared with R27, MS SIM analysis of R32 failed to detect the relatively early eluting delphinidin-3-*O*-acetylglucoside (channel 1, $m/z = 507$). However MS SIM analysis of R32 found two matches which were not detected in R27: 5-(4'-hydroxyphenyl)-pyranomalvidin-3-*O*-acetylglucoside (channel 28, $m/z = 651$) and 5-(4'-hydroxyphenyl)-pyranomalvidin-3-*O*-coumaroylglucoside (channel 29, $m/z = 755$). It is possible that these two very late eluting compounds were not detected in R27 simply because the gradient did not extend for a long enough. This suggests the method used for these runs may require further adjustment to allow very late-eluting pigments to be analysed using 10% formic acid. The 1% and 0.1% formic acid runs demonstrate that in spite of poor peak shapes, MS SIM detection of late-eluting pigments is broadly as successful as with 10% formic acid. The similarity of MS SIM detection results between R27 and R32 also confirms that despite the relatively high acidity using 10% formic acid, MS detection of late-eluting compounds is successful and can be conveniently combined in a single method for the quantitation of the anthocyanin-3-*O*-glucoside pigments.

6 Summary and suggestions for future work

The most important variable impacting the LC/MS resolution of anthocyanins is the type of acid used to acidify solvents A and B, and the concentration used. Although acetic acid at 10% concentration was able to provide baseline separation of Pn-3-Glu and Mv-3-Glu, formic acid was found to produce far better anthocyanin resolutions (cf. R19 (10% acetic acid) Pn-3-Glu / Mv-3-Glu $R_s = 3.36$; R20 (10% formic acid) Pn-3-Glu / Mv-3-Glu $R_s = 14.40$). The peak widths of R19 and R20 were comparable, so the increase in resolution came from a much wider range of retention times for the 5 anthocyanins using formic acid. This is thought to be due to the considerably higher polarity of formic acid resulting in a more polar A solvent and thus greater anthocyanin affinity for the reverse-phase C_{18} column. The concentration of formic acid also affected resolution, with greater acidity markedly improving resolution (cf. R21 (10%) Pn-3-Glu / Mv-3-Glu $R_s = 14.74$; R23 (1%) Pn-3-Glu / Mv-3-Glu $R_s = 5.20$; R24 (0.1%) Pn-3-Glu / Mv-3-Glu $R_s = 2.23$). The main operational disadvantage of using strong acetic or formic acids to increase anthocyanin resolution was the need to purge the pumps and flush the LC column with acid-free solvent at the end of each working day to prevent any degradation of the instrument components.

The importance of solvent gradient choice was demonstrated during the phases of the method development which used 1% acetic acid, a less than optimal acid type and concentration. By replacing an 11% B isocratic elution (R10) with a 5-10% B gradient (R12), Pn-3-Glu / Mv-3-Glu R_s was improved from 0.85 (substantial overlap) to 2.01 (baseline separated) without altering any other LC variables. Once an optimal acid type and concentration was found (10% formic acid), solvent gradient became the most important variable in determining the amount of time taken for anthocyanins to elute. By increasing the solvent B gradient from 0.05% B/min (R20) to 0.33% B/min (R25), the time required for Mv-3-Glu to finish eluting decreased from ca. 43 min to ca. 25 min, whilst still maintaining an excellent Pn-3-Glu / Mv-3-Glu R_s of 9.41.

The remaining factor which improved the Pn-3-Glu / Mv-3-Glu separation factor was oven temperature (cf. R5 (25 °C) Pn-3-Glu / Mv-3-Glu $\alpha = 1.00$; R6 (50 °C) Pn-3-Glu / Mv-3-Glu $\alpha = 1.02$). Increasing the column flow rate above the manufacturer's guideline of 0.2 mL/min did not improve R4 (0.2% acetic acid), however this might be worth revisiting as a way to provide small improvements to an otherwise optimised method. The LC/MS methods developed in this work were successfully applied to the identification and relative quantitation of anthocyanins in four Church Road varietal wine samples.

Future anthocyanin method development could look at increasing the acid polarity further by using trifluoroacetic acid or perhaps even a mineral acid (e.g. HCl) in place of formic acid. Mineral acids are effectively 100% ionised in water and might provide the best possible R_s values by maximising the polarity of the A solvent. The mineral acid concentration would require development from relatively low concentrations, and would need to be considered in terms of the degree of acidity the LC and/or MS instruments are specified to safely handle. The anthocyanin separations obtained using the methods developed in this study are good enough that there remains the potential for further reductions in analysis time through the use of even faster B solvent gradients. The use of internal standards or machine calibration would allow fully quantitative measurements of anthocyanins to be undertaken, rather than the purely relative measurements obtained in the present study.

The late-eluting pigment LC/MS method development resulted in a number of late-eluting pyranoanthocyanins, acylglycosides and an ethyl-bridged dimer being tentatively identified in samples of Pinot Noir (R26) and Church Road Cabernet Sauvignon (R27). Future work could involve using stronger samples or larger injections to provide better detection of the smallest peaks, which in this study were often too small to allow MS SIM identification. A more thorough approach would vary the MS detection voltage to encourage fragmentation of the parent ions in the MS detector. Analysis of the fragmented ions would help differentiate between those candidate molecules with identical parent ion m/z ratios. The UV-Vis spectra of late-eluting pigments could be compared against those presented in the literature to provide a second form of characterisation, although careful attention to solvent pH (and possibly multiple runs with solvents of different pH values) would be required to ensure accurate comparisons.

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Appendix

A spreadsheet containing a summary of the data for all LC/MS runs is attached as a separate file.